THE DEVELOPMENT OF MODEL SYSTEMS TO EXPLORE MECHANISMS OF CHEMICAL STRESS

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor of Philosophy

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DECLARATION

This thesis is the result of my own work. The material contained within this thesis has not been presented, nor is currently being presented, either wholly or in part for any other degree or qualification.

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ABSTRACT

Adverse drug reactions (ADRs) continue to be a major public health issue, accounting for a high incidence in patient morbidity and mortality. Mechanisms of ADRs are complex, therefore drugs often undergo rigorous assessment for their clinical safety, utility and efficacy in man. In doing so, model systems are often employed to simulate the body's response to a particular drug under a certain condition, such as that of a particular disease, for example. However, research in this area is often hampered by the limitations posed by model systems that are currently available, resulting in a gap in predicting the drug's pharmacotoxicology which determines its use man. To bridge this gap, continued research in the development of better model systems is required in order to reduce and manage the clinical consequences of ADRs.

In light of the problem that is currently faced in drug safety research, the purpose of this PhD project was to identify the development of new model systems for investigating the consequences of chemical stress. In particular, this project focussed on investigating the suitability of two independent model systems and a potential biomarker for studying drug-induced liver injury (DILI), given the role of the liver in drug metabolism. The non-tumourigenic human liver epithelial cell line, THLE-2E1, engineered to overexpress the cytochrome P-450 2E1 enzyme was investigated because of the role of CYP2E1 in the toxicological profiles of a number of clinically relevant compounds, including that of alcohol and paracetamol (APAP), as well as its ability to generate oxidative stress through its interaction with NADPH-cytochrome P450 reductase (CPR). The second model evaluated was the nuclear factor erythroid 2-related factor 2 (Nrf2) null mouse ($Nrf2^{(-/-)}$) due to the regulatory role of the transcription factor Nrf2 in a wide array of cytoprotective genes including glutamate cysteine ligase (GCL), the enzyme responsible for the rate limiting step in the synthesis of the antioxidant glutathione (GSH). Having been recently reported as a potential biomarker of GSH consumption, the GSH analogue, ophthalmic acid was also assess for its utility as a translatable biomarker of oxidative stress across *in vitro* and *in vivo* systems, and in man.

The THLE-2E1 cells along with the THLE-Null which contains an empty vector were characterised for morphological difference, CYP2E1 protein content and activity as well as for toxicity in response to paracetamol treatment. These experiments demonstrated no morphological difference but a slower growth rate (42 h) in the THLE-2E1 compared with the THLE-Null cells (29 h). CYP2E1 overexpression and activity (0.67 nmol/mg/protein) were only observed in the THLE-2E1 cell line. Furthermore, GSH content was observed to be higher in the THLE-2E1 (74.89, 50.62 and 37.91 nmol/mg/protein at 24, 48 and 72 h respectively) compared with the THLE-Null (41.98, 35.17 and 29.74 nmol/mg protein at 24, 48 and 72 h respectively), suggesting an up-regulation of cell defence in these cells. Interestingly, treatment with paracetamol did not demonstrate a difference in toxicity between the THLE-2E1 and THLE-Null cells, despite the CYP2E1 activity observed in the THLE-2E1 with the substrates chlorzoxazone (CHZ) and APAP, which was distinctly absent in the THLE-Null. Both the high GSH level and absence of CYP2E1-mediated toxicity in the

THLE-2E1 cells suggests an up-regulation of cellular defence as a consequence of high oxidative stress in these cells generated by the interaction between CYP2E1 and CPR. As well as insufficient CYP2E1 activity, despite overexpression and CYP2E1 activity measured in the THLE-2E1 cells compared with the THLE-Null, demonstrated a similar toxicity profile to the THLE-Null cells. The THLE-2E1 cell line is therefore a potentially useful model of oxidative stress, and to some extent in CYP2E1-mediated metabolism studies, however, this cell line is not suitable for toxicity based investigations.

$Nrf2^{(+/+)}$ and $Nrf2^{(-/-)}$ animals were treated with either an acute dose (350 mg/kg) across a 5 h time point, an escalating chronic dose (0 – 600 mg/kg) for 8 days or an escalating chronic dose (0 – 600 mg/kg; 8 days) with a further toxic dose challenge (1000 mg/kg; day 9) of paracetamol to determine the role of Nrf2 in the regulation of the GSH system, which comprises the GSH synthetic pathway and several other metabolic pathways that are closely linked to GSH synthesis. Under the three dosing regimens with paracetamol, the $Nrf2^{(-/-)}$ mice were highly susceptible to paracetamol-induced toxicity as demonstrated by a marked increase in serum alanine transaminase (ALT) activity as well as fatalities in the $Nrf2^{(-/-)}$ group that were chronically-treated and subsequently challenged with a toxic dose of paracetamol. However, this enhanced susceptibility in the $Nrf2^{(-/-)}$ to toxicity was only observed upon reaching a certain threshold of stress as demonstrated in the acute study where a progressive increase in serum ALT was observed at 5 h whilst levels remained the same in the $Nrf2^{(+/+)}$, suggesting the abrogated ability of the $Nrf2^{(-/-)}$ to up-regulate cellular defence. This was further demonstrated by the delay in GSH recovery in the $Nrf2^{(-/-)}$ animals compared with the $Nrf2^{(+/+)}$ under all three dosing regimens. Autoprotection against a toxic dose of paracetamol was demonstrated in the $Nrf2^{(+/+)}$ with lower levels of serum ALT compared with animals that only received the toxic dose. This was also demonstrated in the equivalent $Nrf2^{(-/-)}$ animals but to a lesser extent as more fatalities occurred in this group compared with the wild type. No significant differences were observed in the metabolites measured in the GSH system between the $Nrf2^{(+/+)}$ and $Nrf2^{(-/-)}$ mice. However, a delay in the synthesis of GSH and its analogue, ophthalmic acid (OA), both of which share the same synthetic enzymes, glutamate cysteine ligase (GCL) and glutathione synthetase (GS), and which are both under Nrf2 regulation was observed, therefore providing further support in the abrogated ability of the $Nrf2^{(-/-)}$ mice to up-regulate cellular defence in response to chemical stress. This piece of work demonstrates that the $Nrf2^{(-/-)}$ mouse is a useful model of chemical stress as Nrf2 is clearly an important determinant in cytoprotection against chemical stress. However, the similarities in response observed between the two strains also suggest that other mechanisms are involved particularly in the acquirement of autoprotection against a toxic APAP dose. Due to the role of Nrf2 in cellular defence, work carried out here have focused on cytoprotection against chemical stress, however, other changes in metabolic activity should also be investigated in these animals as they are responsible for the production of chemical stress in the first instance.

Mouse and rat treated with APAP and diethyl maleate (DEM) respectively were measured for liver and/or serum ophthalmic acid, GSH and ALT levels to determine if OA is a direct biomarker of GSH consumption. The mouse hepatoma cell line Hepa1c1c as well as the human THLE-Null and THLE-2E1 cells were treated with either DEM, the APAP reactive

metabolite, N-acetyl-*p*-benzoquinoneimine (NAPQI) or paracetamol, and were also measured for cellular GSH and OA levels to determine the translatability of OA as an oxidative stress biomarker across *in vitro* and *in vivo* systems using a variety of GSH modulators. This was further explored in man where basal OA in human serum was also assessed. A significant increase in liver and serum OA over time in response to APAP was observed in the APAP-treated mice but was not concurrent with GSH depletion. Furthermore, although a statistically significant depletion in GSH was observed in rats treated with DEM, no corresponding increase in hepatic or serum OA was observed. None of the experiments carried out *in vitro* demonstrated a correlation between GSH depletion and ophthalmic acid increase, suggesting that OA is not a direct marker of GSH depletion. However, where an increase in ALT was observed in any of these experiments, OA levels were also consistently elevated, suggesting that OA may be a general marker of chemical-induced toxicity. The ability to detect and quantify extremely low levels of basal OA in human serum was demonstrated, and was observed to exhibit both intra- and inter-individual variation, similar to that observed with ALT. Ophthalmic acid is not a direct biomarker of GSH consumption but its potential as a general marker of toxicity should be further explored. The ability to detect and quantify basal OA in human serum are important attributes of a biomarker, particularly in man, therefore the role of OA in chemical stress should be further investigated as OA may have the potential as a marker of chemical stress in man.

To conclude, the models investigated in this project have the potential as models for use in the preclinical evaluation of chemical stress. However, none provides all the attributes needed to address all toxicological issues. As such, this reiterates the difficulty in the development of better model systems, therefore continued research in this area should be encouraged.

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PUBLICATIONS

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ABBREVIATIONS

2-AB	2-amino butyrate
2-KB	2-ketobutyrate
ADR	adverse drug reaction
AKS-1	apoptosis signal-regulating kinase 1
ALF	acute liver failure
ALP	alkaline phosphatise
ALT	alanine aminotransaminase
ALT-1	alanine transaminase isoform 1
ALT-2	alanine transaminase isoform 2
ANOVA	analysis of variance
AP-1	activating protein-1
APAP	acetaminophen/APAP
ARE	antioxidant response element
AS	APAP:saline
AST	aspartate transaminase
ATP	adenosine triphosphate
BHMT	betaine:homocysteine methyltransferase
BPE	bovine pituitary extract
BSA	bovine serum albumin
Ca^{2+}	calcium
cAMP	cyclic adeonise monophosphate
CBP	CREB-binding protein
CBS	cystathionine β-synthase
CCl_4	carbon tetrachloride
cDNA	complementary DNA
cDMEM	complete DMEM
CDO	cysteine dioxygenase
CF	conversion factor
CM	complete media
CMV	cytomegalovirus
CNC	cap'n'collar
CO_2	carbon dioxide
COX	cyclooxygenase
CPR	NADPH cytochrome-P450 reductase
CRE	cyclic adeonise monophosphate-responsive element
CSD	sulfinate decarboxylase
CUL3	cullin 3
CYP1A2	cytochrome P450 1A2 isoform
CYP2E1	cytochrome P450 2E1 isoform
CYP3A4	cytochrome P450 3A4 isoform
CYP450	cytochrome P450
Cys	cysteine
dcSAM	decarboxylated SAM
DF	dilution factor
dH_2O	distilled water

DILI	drug-induced liver injury
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl suloxide
DNA	deoxyribonucleic acid
DTNB	5,5'-dithiobis(20bitrobenzoic acid)
EDTA	ethylendiaminetetraacetic acid
EGF	epidermal growth factor
EPH	epoxide hydrolase
FasL	Fas ligand
FBS	fetal bovine serum
FDA	food and drug administration
FMO	flavin-containing monooxygenase
g	gram
GCL	glutamate cysteine ligase
GCLC	glutamate cysteine ligase catalytic subunit
GCLM	glutamate cysteine ligase modulator subunit
GPx	glutathione peroxidase
GS	glutathione synthetase
GSH	reduced glutathione
GSSG	oxidised glutathione
GSTP1-1	GST subunit P1-1
GSTs	glutathione-*S*-transferases
h	hour
H_2O_2	hydrogen peroxide
HBSS	hanks balanced salt solution
HepG2	human hepatocellular liver carcinoma cells
HF-1	hypoxia inducible factor 1
HL-hep	cryopreserved human liver hepatocyte
HL-hom	human liver hhomogenate
HMGB1	high-mobility group protein 1
HO-1	heme oxygenase 1
HPLC	high performance liquid chromatography
HSF	heat shock factor
i.p.	intraperitoneal
IL-10	interleukin 10
IL-1B	interleukin 1B
iNOS	inducible intrinsic oxide synthase
ITS	insulin-transferrin-sodium selenite supplement
KCl	Postassium chloride
Keap1	Kelch-like ECH-associated protein
kg	kilogramme
Ki	dissociation constant
Km	rate constant
L	litre
LC-MS-MS	liquid chromatography tandem mass spectrometry

m/z	mass-to-charge ratio
MAO	monoamine oxidase
MAT	methionine adenosyl transferase
MDH	malate dehydrogenase
MeOH	methanol
mg	milligramme
Mg^{2+}	magnesium
Min	minute
mM	millimolar
MOPS	3-(N-morpholino)propanesulfonic acid
MRM	multiple reaction monitoring
mRNA	messenger ribonucleic acid
MRP	multidrug resistam protein
MS	mass spectrometry
MS-MS	tandem mass spectrometry
MTS	(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)
NaCl	sodium chloride
NADPH	nicotineamide adenine dinucleotide phosphate
NaH_2PO_4	Sodium phosphate
NaOH	Sodium hydroxide
NAPQI	N-acetyl-*p*-benzoquinoneimine
NAT	N-acetyl-transferase
NF-kB	nuclear factor kB
nm	nanometre
nmol	nanomole
NQO1	NAD(P)H:quinine oxidoreductase 1
Nrf2	nuclear factor erythroid 2-related factor 2
$O^{2.-}$	superoxide anion
OH	hydroxyl group
P-gp	P-glycoprotein
PNC	phenacetin
PRX	peroxiredoxin
RIPA	radioimmunoprecipitation assay
ROS	reactive oxygen species
rpm	revolutions per minute
SA	saline:APAP
SAH	S-adenosyl homocysteine
SAM	S-adenosyl methionine
SAMDC	SAM decarboxylase
SD	standard deviation of mean
SEM	standard error of mean
SFM	serum free media
SOD	superoxide dismutase
SS	saline:saline
SSA	sulfosalacylic acid
SSAT	spermidine/spermine N-acetyltransferase

SSRI	serotonin-selective uptake inhibitors
SULT	sulfotransferase
SV40	Simian vacuolating virus 40
TauT	taurine transporter
TBS	tris-buffered saline
THLE	non-tumourigenic human liver epithelial cells
TNF-α	tumour necrosis alpha
TPA	12-o-tetradecanoate-13-acetate
TRE	TPA-responsive element
TRX	thioredoxin
T-TBS	tween-TBS
U	unit
UDPGA	uridine diphosphate glucuronic acid
UGT	UDP-glucuronosyltransferases
ULN	upper limit of normal
USA	United States of America
v/v	volume/volume
w/v	weight/volume
γ-Cys	γ-cystathionase
γ-GC	γ-glutamylcysteine
γ-GGT	γ-glutamyl transpeptidase
γ-Glu-2AB	γ-2-aminobutyrate
μ	micron
μl	microlitre
μM	micromolar

Chapter 1

General Introduction

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1.1 INTRODUCTION

Adverse drug reactions (ADRs) are major contributors to the high incidence of patient morbidity and mortality (Park *et al.*, 2005a, Pirmohamed *et al.*, 2004), and as a consequence, numerous drugs have been withdrawn either at the development or post marketing stage (Jefferys *et al.*, 1998). Drug-induced liver injury (DILI) in particular, is the most prevalent type of ADR due to the role of the liver in drug metabolism (Park *et al.*, 1995). Indeed, APAP-induced liver injury accounts for a significant proportion of acute liver failure (ALF) in the US (Lee, 2004, Larson *et al.*, 2005) and in Europe (Davern *et al.*, 2006). As such, significant effort has been made by various groups to fully elucidate the mechanism(s) of ADRs. Information obtained so far from these investigations has in turn been utilised for the design and development of better drugs through:

- Continued improvement and development of model systems to gain a better insight into the toxicological nature of xenobiotics and the role of metabolism and transporters, as well as the protective mechanisms adopted by various species to combat against such insults.
- The identification of biomarkers that could be utilised both in research and in the clinic to predict or even prevent the onset of ADRs.
- The identification of structural chemical alerts that have the potential to cause ADRs.

The constant exposure of cells to stress of varying origin has led to the evolution of a multi-faceted system of cytoprotection within the cell, levels of which can be regulated depending on the gravity of stress present. Cell defence has therefore evolved into a set of highly intricate and tightly regulated response mechanisms against cellular stress of varying magnitude. One such mechanism involves the transcription factor Nrf2, which is involved in the transcriptional up-regulation of a variety of cytoprotective genes including glutamate cysteine ligase (GCL), the enzyme responsible for the rate limiting step in the synthesis of the antioxidant glutathione (GSH), when cellular stress is elevated as a consequence of xenobiotic-induced injury for example.

The knowledge obtained from mechanistic investigations on ADRs can therefore be highly valuable in developing model systems such as the identification of novel biomarkers that could be used for the design and development of safer drugs amongst other applications, offering great benefit to both the public and pharmaceutical industry. The continuous development and improvement of model systems should therefore be

encouraged as it is an important aspect in understanding the cause of ADRS and in the design and development of safer drugs.

The focus of this thesis is on the development of novel experimental models in which to investigate the mechanistic basis of DILI and the adaptive response to chemical stress, and the use of such model systems in the pursuit of more sensitive and earlier biomarkers of chemical stress.

1.2 ADVERSE DRUG REACTIONS

Adverse drug reactions, as defined by Edwards and Aronson (2000), are 'an appreciably harmful or unpleasant reaction, resulting from an intervention related to the use of a medicinal product, which predicts hazard from future administration and warrants prevention or specific treatment, or alteration of the dosage regimen, or withdrawal of the product.' In the US, a total of 249, 633 suspected ADRs, based on administrative data, were reported for both ambulatory care and hospitalised patients between 2002 and 2005 (Kane-Gill *et al.*, 2010). Analysis of these data revealed antineoplastic and anticoagulant agents to be the highest risk, followed by antihypertensives, antiarteriosclerotic and antirheumatic agents, being the more common cause of ADRs. ADRs have also proven a costly impediment in drug development and even in the success of those already approved for public administration (Smith and Schmid, 2006). As such, a collaborative effort from drug and health-based organisations of both public and private sectors must therefore be pre-requisites, if the ongoing problem posed by ADRs is to be successfully managed and eventually solved.

Due to the complex nature of ADRs, stringent classification of these reactions is not possible, however, for the sake of general reference, they have been grouped into six main categories as summarised in Table 1.1 (Edwards and Aronson, 2000, Park *et al.*, 2000). Alternatively, ADRs are also regarded as either 'on target' or 'off target' reactions. 'On target' adverse reactions refer to those that could be predicted from the known pharmacology of the drug and in most cases, are resolved with the adjustment of drug dosage. 'Off target' adverse reactions on the other hand, are unpredictable and although occur less frequently, may be life threatening in their severity. One factor that is currently under investigation is differences in inter-individual susceptibility to ADRS as a consequence

of our unique individual genetic make-up (Park *et al.*, 2005a, Wijnen *et al.*, 2007, van Schaik, 2008, Pirmohamed, 2010). In particular, polymorphisms in both phase I and phase II metabolising enzymes such as the cytochrome P450 (CYP450) and UDP-glucuronosyltransferase (UGT) enzyme families have been well documented as important influencing factors in our susceptibility to ADRs (Guillemette, 2003, Carlini *et al.*, 2005, Bauman *et al.*, 2006, Falzoi *et al.*, 2010, Tohkin *et al.*, 2010).

Table 1.1. Classification of adverse drug reactions. Adapted from Park *et al.*, 2005a and Edwards and Aronson, 2000.

Reaction type	Description	Examples
A (augmented)	Predictable from the known pharmacology of the drug. It is commonly observed but with a low mortality rate.	Digoxin toxicity; serotonin syndrome with serotonin-selective reuptake inhibitors (SSRIs).
B (bizarre)	Unpredictable from the known pharmacology of the drug (idiosyncratic). It is less common but proves to be serious and fatal.	Penicillin hypersensitivity; malignant hyperthermia (exposure to general anaesthesia); pseudoallergy (ampicillin rash).
C (chemical)	Their biological activities can often be predicted or rationalised from their chemical structure.	APAP toxicity.
D (delayed)	Adverse effects are not apparent until after a certain period of drug use.	Carcinogenicity, teratogenicity.
E (end of treatment)	Occurs upon the withdrawal of a drug either at the end or as an abrupt termination of a treatment plan.	Withdrawal of phenytoin and paroxetine; opiate withdrawal syndrome; myocardial ischaemia (β-blocker withdrawal).
F (failure)	Dose-related and often caused by drug interactions.	Inadequate dosage of a contraceptive particularly when used with specific enzyme inducers.

1.3 THE LIVER

All drugs have the potential to cause toxicity (Halegoua-De Marzio and Navarro, 2008). The liver, rich in metabolising enzymes, is the main organ where biotransformation and subsequent detoxification of xenobiotics including drugs take place. As such, the liver is constantly exposed to high levels of parent drugs and metabolites (chemically stable and/or reactive) produced during drug bioactivation. As a consequence, the liver is a primary target for drug-induced toxicity. Indeed, DILI is the most common ADR, resulting as a major burden in patient health (Kaplowitz, 2005, Davern *et al.*, 2006) and a costly obstacle in the pharmaceutical industry (Park *et al.*, 2005b, Antoine *et al.*, 2008). As such, the liver is an important focal point in the study of ADRs.

1.4 DRUG METABOLISM

Essentially, the role of drug metabolism is to eliminate drugs from the body. This is achieved through the conversion of non-polar, lipophilic compounds to polar, hydrophilic entities, via enzymatically-driven reactions, reducing their ability to penetrate tissues and renal tubular reabsorption. Drug metabolism is more commonly divided into two distinct, often sequential phases, phase I and phase II as summarised in Table 1.2. However, a more recent category referred to as phase III, which is concerned with the transport of parent drugs and their respective metabolites across cell membranes has been included and is also summarised in Table 1.2.

Table 1.2 Phases I, II and III of drug metabolism.

	Phase I	Phase II	Phase III
Function(s)	Addition of functional groups for phase II reactions. Activating intermediary process employed by prodrugs.	Addition of endogenous molecules (glucuronic acid, GSH, sulphate, amino acid and acetate) to increase the hydrophilicity of drugs/metabolites.	Provide barrier against drug penetration. Key player in drug absorption, distribution and excretion.
Reaction(s)	Oxidation, reduction and hydration.	Sulfation, glucuronidation, acetylation and GSH conjugations.	ATP-dependent substrate transport. Sodium- and ATP-independent transport.
Key proteins	Cytochrome P450 (CYP) microsomal enzymes. NAD(P)H:quinone oxidoreductases (NQO1), Epoxide hydrolases (EPH), Flavin-containing monooxygenase (FMO), cyclooxygenase (COX), monoamine oxidases (MAO) and hydrolases.	Sulfotransferase (SULT), UGTs, GSTs and N-acetyltransferases (NAT).	P-glycoprotein (P-gp) Multidrug resistance-associated protein (MRP) Organic anion transporting polypeptide 2 (OATP2).

Despite the intended benefit of drug metabolism to the body, accumulation of the parent drug and/or highly reactive species (reactive oxygen species (ROS) and electrophiles) produced as a consequence of drug bioactivation can sometimes overwhelm the cell's defence system, after a drug overdose for instance (unintended or not), or due to inter-individual pharmacogenetic susceptibility to a drug (Tohkin *et al.*, 2010). Such a situation could lead to unmanageable levels of ROS and electrophiles increasing the potential for protein adduct formation and oxidation. This in turn may result in the accumulation of protein damage, cell death, organ toxicity and even death (Figure 1.1).

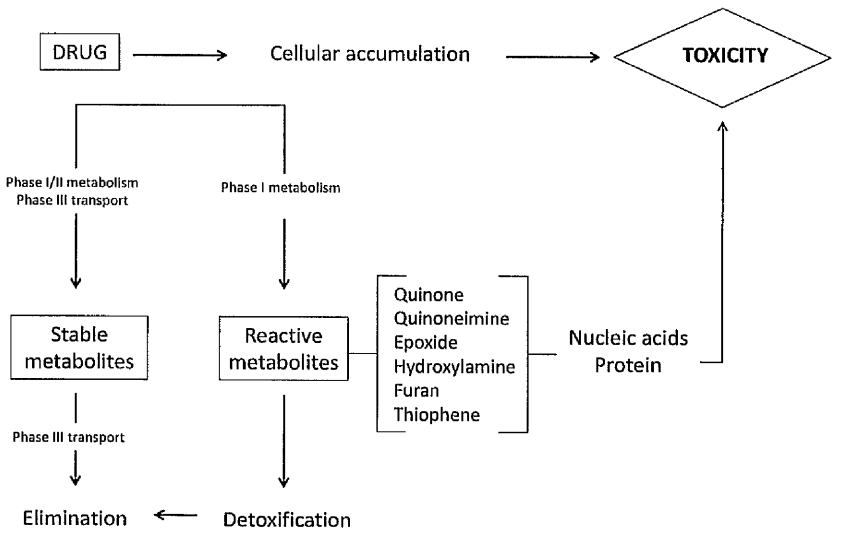


Figure 1.1 The role of drug metabolism in adverse drug reactions. Under normal circumstances, drugs undergo phases I, II and III metabolism, all of which aim to convert lipophilic molecules into more hydrophilic entities facilitating their safe elimination from the body. Drug metabolism often involves drug bioactivation which results in the production of reactive metabolites, which are normally deactivated through conjugation with GSH. However, when levels of these reactive species surpass the cell's ability to detoxify them at a sufficient rate, macromolecules become at risk of becoming targets for covalent binding and oxidation, damages which if unrepaired and accumulate, could lead to toxicity. Adapted from (Park *et al.*, 2000, Copple *et al.*, 2008b).

1.5 DRUG-INDUCED LIVER INJURY

Drug-induced liver injury is a rare event with its true incidence still remaining undetermined, however, its clinical importance as the most prevalent cause of acute liver failure signifies its relevance as an important safety issue in public health (Ostapowicz *et al.*, 2002). Furthermore, DILI has led to the withdrawal of 800 drugs to date, a major burden which carries great cost to the pharmaceutical industry (Antoine *et al.*, 2008).

DILI is regarded as either predictable or idiosyncratic (Pachkoria *et al.*, 2007), although the majority manifest as the latter, attributing to 13 – 17 % of acute liver failure (Bjornsson and Olsson, 2005). Idiosyncratic DILI may also have a dose-dependent component and often occurs after a latent period of up to one year (Lee, 2003, Abboud and Kaplowitz, 2007), reiterating the unpredictability of this disease. Whilst statin-induced DILI are often resolved and have been rarely associated to cause idiosyncratic DILI, antibiotics and NSAIDS are the most common causative drugs along with antiretroviral therapy which is reported to cause DILI in 10 % of patients under therapy (Blinkova *et al.*, 2007). APAP is a

classic example of predictable DILI (section 1.5.1), where its toxicity is induced in a dose-dependent manner, usually after an overdose (Laine *et al.*, 2009). Susceptibility to the development of DILI encompasses a combination of both environmental and intrinsic factors including age, sex, genetic polymorphisms in metabolising enzymes and pre-existing liver disease as summarised in Table 1.3.

Table 1.3 A summary of factors that influence the susceptibility of an individual to develop DILI.

Factor	Example
Age	Co-amoxiclav and isoniazid hepatotoxicities are more common in older individuals whilst sodium valporate and erythromycin hepatotoxicities are more prevalent in young people (Maddrey, 2005).
Sex	Female patients accounted for 76 % of liver transplant recipients for the treatment of drug-induced acute liver failure between 1990 and 2002 (Morgan *et al.*, 1984, Ostapowicz *et al.*, 2002, Russo *et al.*, 2004). Sex hormones and pro-inflammatory cytokines can alter liver metabolism which influences the susceptibility between gender to acute DILI (Flynn and Ferguson, 2010).
Genetic polymorphism	CYP2D6 deficiency has been reported to be the cause of perhexiline hepatotoxicity (Morgan *et al.*, 1984, Alvan *et al.*, 1990). Variants within the HLA complex have been associated with flucoxacillin-induced (Daly *et al.*, 2009), lumiracoxib-induced (Singer *et al.*, 2010) and ximelagatran-induced (Kindmark *et al.*, 2008) liver injuries.
Disease	Pre-existing liver disease increases the susceptibility of HIV and tuberculosis (TB) patients co-infected with hepatitis B or C to the development of DILI (Kontorinis and Dieterich, 2003, Sulkowski, 2003, Sulkowski, 2008).

DILI can either lead to hepatocellular, cholestatic or mixed liver injury. Hepatocellular injury is characterised by marked elevations in alanine-aminotransaminase (ALT) and aspartate-aminotransaminase (AST) enzymes, both of which are highly abundant in the liver and are therefore believed to be hepatic leakage products during liver injury (Boyd, 1983, Sherman, 1991, Dufour *et al.*, 2000a, Dufour *et al.*, 2000b). Other markers of liver injury are also observed including increases in total plasma bilirubin and alkaline phosphatase (ALP), although for hepatocellular-specific injury, they are preceded by ALT

and AST elevations in the serum. Cholestatic injury on the other hand is characterised by the opposite, with a significant rise in ALP preceding elevations in both ALT and AST (Halegoua-De Marzio and Navarro, 2008). Mixed DILI represents cases that show characteristics of the former two. Although rare, DILI can also manifest as immune-mediated and is characterised by fever, rash, eosinophilia, auto antibodies and antihapten antibodies. Another rare DILI that leads to mitochondrial injury is characterised by significant levels of lactic acid and microvesicular steatosis in liver biopsies.

1.5.1 APAP hepatotoxicity

APAP is a clinically relevant model of DILI (Ostapowicz *et al.*, 2002). It is tolerated at therapeutic doses but is often fatal when taken at an overdose. APAP is primarily metabolised through conjugation reactions with SULT and UGT enzymes. However, a small proportion can also undergo CYP450-mediated oxidation, in particular, by CYP2E1, CYP1A2 and CYP3A4 isoforms, to the reactive intermediate, N-acetyl-*p*-benzo-quinoneimmine NAPQI (Raucy *et al.*, 1989). At therapeutic doses, NAPQI can be deactivated by conjugation with the antioxidant GSH via the activity of glutathione *S*-transferases (GSTs). However, when levels of NAPQI overwhelms the rate of detoxification resulting in the depletion of GSH store, NAPQI becomes free to bind to cellular nucleophiles causing cell damage and even cell death (Jollow *et al.*, 1973, Mitchell *et al.*, 1973a, Mitchell *et al.*, 1973b, Potter *et al.*, 1973, Dahlin *et al.*, 1984, Lee *et al.*, 1996). The production of NAPQI combined with the inability of the cell to sufficiently deactivate overwhelming levels of this reactive intermediate, during an overdose for example, are two main determinants of APAP-induced toxicity. Indeed, reports of APAP toxicity have been made from a therapeutic dose (Moling *et al.*, 2006, Dart and Bailey, 2007), however, this is often in conjunction with other underlying conditions which predisposes an individual to APAP toxicity, including chronic alcohol consumption (Whitcomb and Block, 1994, Prescott, 2000, Schmidt *et al.*, 2002, Krahenbuhl *et al.*, 2007) and malnutrition (Whitcomb and Block, 1994).

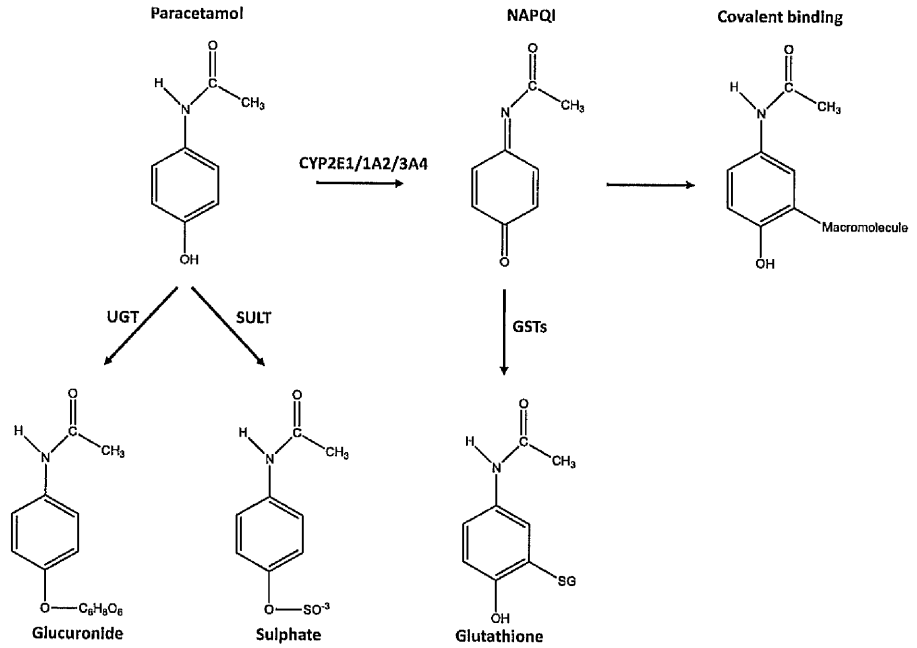


Figure 1.2 The metabolic fate of APAP. APAP (paracetamol) is primarily metabolised by sulfotransferases (SULTs) and UDP-glucuronosyl transferases (UGTs) and subsequently excreted in the urine. A small proportion can also undergo phase I metabolism via CYP2E1, CYP1A2 or CYP3A4 oxidation into the reactive intermediate N-acetyl-*p*-benzo-quinoneimine (NAPQI), which can be deactivated through GSH conjugation mediated by glutathione-*S*-tranferases (GST), forming a mercapturate which is also excreted in the urine. However, bioactivation of high levels of APAP to NAPQI, during an overdose for instance, may overwhelm cellular defence, resulting in the depletion of GSH therefore allowing NAPQI to bind to macromolecules causing cell damage and cell death.

1.6 CELLULAR DEFENCE

To protect against the constant exposure to hazardous, potentially toxic molecules from both endogenous and exogenous origin, the liver has evolved a multi-layered defence system that is designed to combat cellular stress in a variety of situations as detailed in sections 1.6.1 – 1.6.3 and summarised in Figure 1.4. However, although such defence system exists, an overwhelming increase in cellular stress as a consequence of exposure to high levels of drugs for example may lead to insufficiency in the safe clearance of drugs as described in section 1.5.1, disrupting the balance between cellular stress and defence (Figure 1.3). Reactive species that could not be deactivated are therefore free to cause cellular damage through protein covalent binding and oxidation potentially resulting in toxicity.

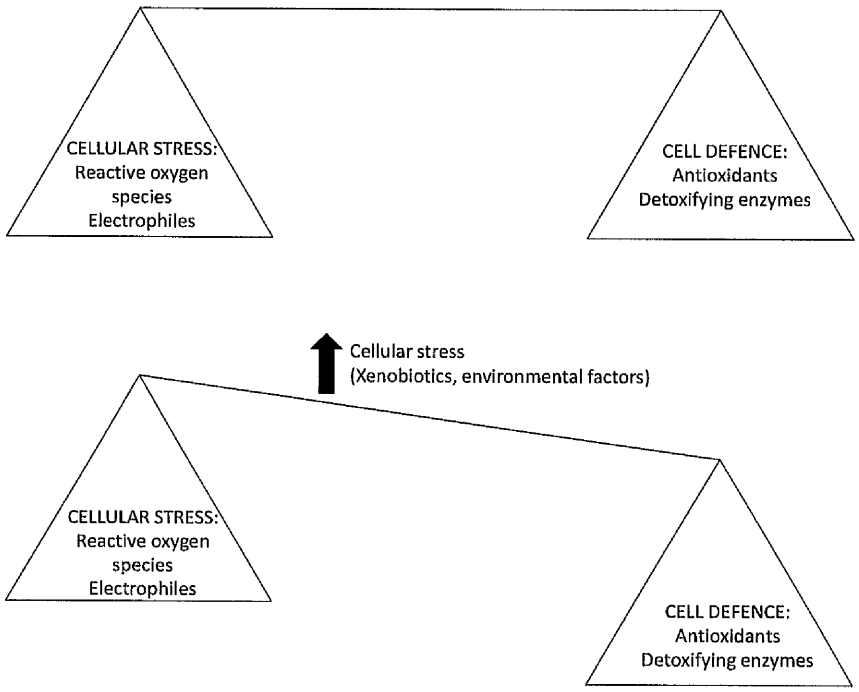


Figure 1.3 The disruption of balance between cellular stress and cell defence as a consequence of significant elevations in cellular stress.

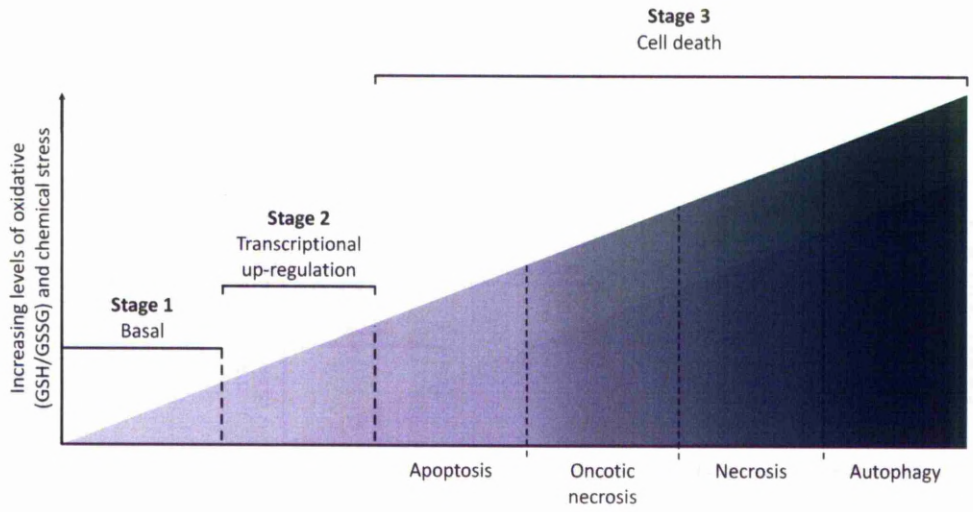


Figure 1.4 Classification of cellular defence. The level of cellular defence is determined according to the severity of cellular stress present. Stage I refers to basal cytoprotection against intrinsic, low levels of oxidative stress. Transcriptional up-regulation of antioxidants and detoxifying enzymes constitutes stage II and is induced upon a considerable increase in cellular stress, often as a result of a combination of internal and external factors (such aas genetic polymorphism and smoking respectively for example). The transcription factor Nrf2 plays an important role in the transcriptional up-regulation of cell defence genes both at stages I and II as detailed further in section 1.6.2.1. Stage III involves cell death which can be classified into four stages, apoptosis, oncotic necrosis, necrosis and autophagy. For a more detailed description of each of these cell death mechanisms, please refer to (Edinger and Thompson, 2004, Duprez *et al.*, 2009, Malhi *et al.*, 2010). The cell enters stage III when the extent of cell damage is so great that the sacrificial death of one or a small population of cells proves the best strategy in keeping the integrity of neighbouring cells and the organism as a whole. Adapted from (Nel *et al.*, 2006).

1.6.1 Stage I: Basal antioxidants and detoxifying enzymes

Reactive oxygen species (ROS), although potentially toxic, are inherently produced through various aerobic metabolic reactions in the cell (Yu, 1994). Furthermore, ROS are now becoming more accepted to have an essential role in a variety of normal cell functions including intracellular signalling, regulation of transcription factor activities, gene expression and redox status (Palmer *et al.*, 1987, Furchgott, 1995, Sundaresan *et al.*, 1995, Finkel, 1998, Kamata and Hirata, 1999, Rhee, 1999, Patel *et al.*, 2000, Bogdan, 2001). However, they can also readily react with macromolecules including DNA (Mates *et al.*, 1999, Marnett, 2000), lipids (Halliwell, 1993, Steinberg, 1997, Frei, 1999, Yla-Herttuala, 1999) and proteins (Butterfield *et al.*, 1998, Stadtman and Berlett, 1998) that may lead to cellular damage and eventually cell death. As such, ROS levels are tightly regulated by closely coupling their utility in various cellular processes with their detoxification to prevent

uncontrollable levels to escalate. Protective antioxidant mechanisms have therefore evolved to combat against basal oxidative stress and are grouped into non-protein and protein molecules.

1.6.1.1 Non-protein antioxidants

Non-protein antioxidants deactivate both ROS and electrophiles in a sacrificial manner by donating an electron to radical species, thus deactivating the reactive nature of such species. Such antioxidants include ascorbic acid (Sies *et al.*, 1992, Buettner, 1993, Meister, 1995), α-tocopherol (Chopra and Thurnham, 1999, Yla-Herttuala, 1999), β-carotene and bilirubin (Yu, 1994).

1.6.1.2 Glutathione

Glutathione (GSH) is a non-protein thiol that is ubiquitously found in mammalian cells with a particular abundance in hepatocytes (5 – 10 mM). It is a tripeptide (γ-glutamyl-cysteinyl glycine) that is synthesised in a two step process (Figure 1.5). The first step, which involves the conjugation of glutamate and cysteine, catalysed by glutamate-cysteine ligase (GCL) to form γ-glutamyl cysteine (γ-GC), is also the rate-limiting step of this pathway. Subsequent conjugation of γ-GC with glycine through the catalytic activity of glutathione synthetase (GS) completes the synthesis of GSH (Bloch and Anker, 1947, Meister and Anderson, 1983).

Under physiological conditions, its principal role is to maintain the cell's redox status by undergoing thiol-disulfide exchange reactions catalysed by thiol-transferases (Carlberg and Mannervik, 1985). This reversible reaction allows an equilibrium to be reached, so that the oxidised form, GSSG, is kept to a minimum, with levels maintained to approximately 1 % of its reduced (GSH) counterpart. GSH plays an important role in cellular defence in that ROS produced either from normal aerobic metabolism or chemical stress, as well as electrophiles, are deactivated by GSH directly or through enzymatic reactions catalysed by various detoxifying enzymes including glutathione sulfotransferases and peroxidises (Awasthi *et al.*, 1975, Sies *et al.*, 1997). The resulting GSH conjugates are either exported from the cell into bile or blood where they may undergo subsequent cleavage of the γ-glutamyl moiety by γ-glutamyl transpeptidases (GGT) and the cysteinyl-glycine bond by dipeptidases. The resulting cysteinyl conjugate undergoes N-acetylation and forms a

mercapturate that is excreted in the urine (DeLeve and Kaplowitz, 1991). In addition, GSH is also implicated in other cellular activities including DNA and protein syntheses, enzyme activity, transport, cell proliferation and as a store of the highly important amino acid, cysteine (Cho *et al.*, 1981, Meister and Anderson, 1983, Wu *et al.*, 2004, Townsend, 2007).

As a consequence of its multiple roles, maintenance of GSH levels is crucial for the survival of the cell both physiologically and during cellular stress. As such, various mechanisms have been employed by the cell for regulating the GSH pool. One such mechanism is the rate-limiting step in GSH synthesis where GCL activity is up-regulated when cellular GSH is severely depleted. Conversely, high levels of cellular GSH negatively regulate GCL activity thereby preventing further GSH synthesis (Richman and Meister, 1975, Griffith and Meister, 1979, Maede *et al.*, 1982, Reed and Fariss, 1984, Russo *et al.*, 1986, Tsutsui *et al.*, 1986, Furukawa *et al.*, 1987, Coursin and Cihla, 1988, Meister, 1988, Sun *et al.*, 1988). GCL is a heterodimer that is made up of the subunits, glutamate cysteine ligase catalytic (GCLC) and glutamate cysteine ligase modulator (GCLM). GCLC is responsible for the enzyme's catalytic activity (binds the substrates) whilst GCLM is involved in regulating this catalytic activity (enhances the binding activity by lowering the Km for glutamate and increasing the Ki to GSH feedback inhibition) (Meister, 1983, Griffith, 1999, Yang *et al.*, 2007). The interaction of these two subunits is therefore an influencing factor on the activity of GCL, hence GSH synthesis (Meister and Anderson, 1983, Griffith, 1999, Yang *et al.*, 2007). Another factor that influences the synthesis of GSH is the availability of its precursors. Although GSH is made up of the three amino acids, cysteine, glutamate and glycine, its role in cellular defence heavily relies on the thiol group of cysteine (Liu *et al.*, 1996, Griffith, 1999, Griffith and Mulcahy, 1999). As such, the availability of cysteine is essential for the synthesis of a fully functioning GSH.

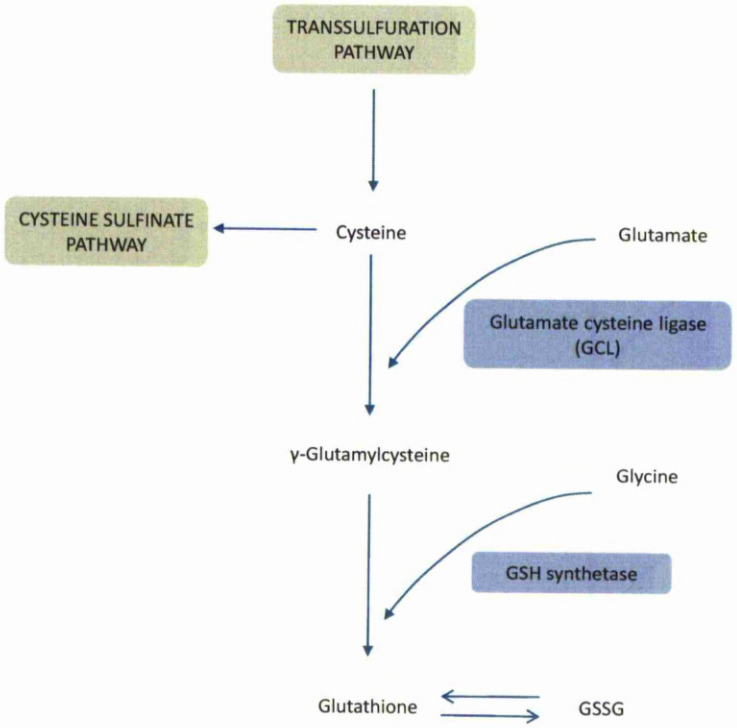


Figure 1.5 The glutathione biosynthetic pathway.

1.6.1.3 The glutathione system

Due to the multi-functional role of GSH in various cellular processes including cell defence, its synthetic pathway is closely linked to several metabolic pathways, some of which are important sources of precursors required for GSH synthesis. Perturbations in these pathways may therefore significantly affect mechanisms by which GSH levels are regulated. Metabolic pathways that are directly linked to the GSH biosynthetic pathway are summarised in Figure 1.6 and individually illustrated and described in Appendices 1 – 4. Ophthalmic acid biosynthesis is introduced in more detail in section 1.6.1.4 and illustrated in Figure 1.7.

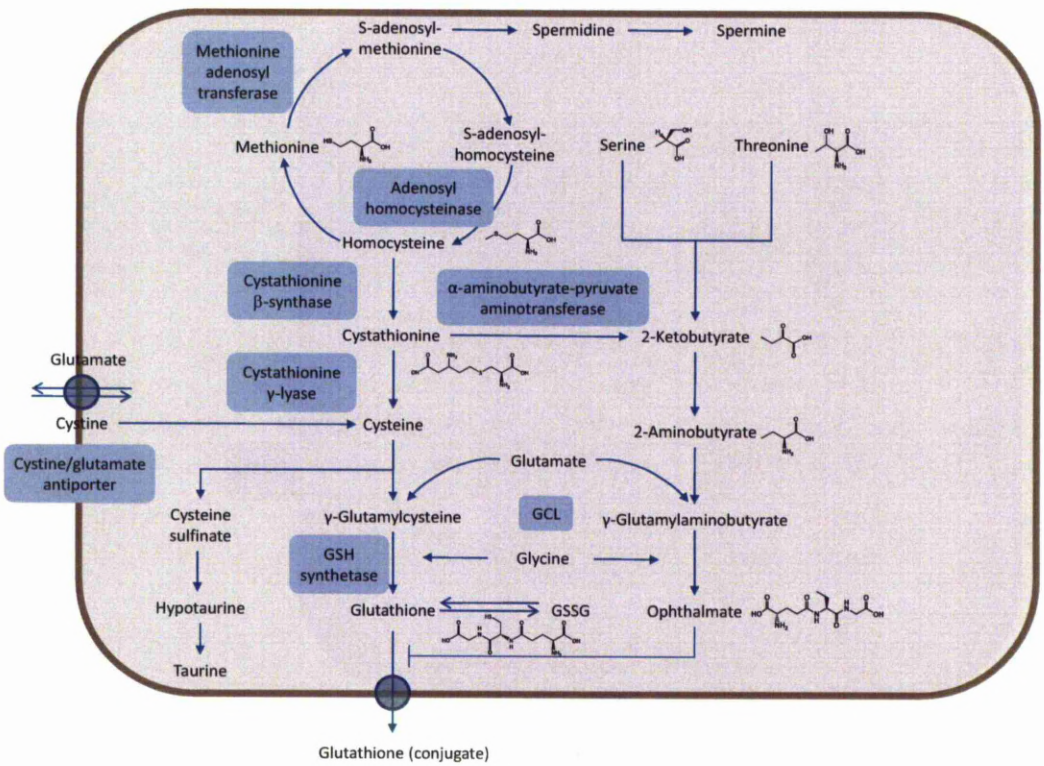


Figure 1.6 The GSH system. A schematic diagram describing the relationship between the GSH synthetic pathway with other closely related metabolic pathways including ophthalmic acid, methionine, transsulfuration, taurine and polyamine pathways as well as the cystine/glutamate transport. Labels in blue box represent enzymes responsible for the catalysis of the reaction in which they are labelled in the system. For a more detailed description of each pathway, refer to Appendices 1 – 4. Labels including GSH, GSSG and GCL are abbreviations used for glutathione, oxidised glutathione and glutamate cysteine ligase respectively.

1.6.1.4 Ophthalmic acid pathway

The functional role of ophthalmic acid is poorly defined. As an analogue of GSH, its use in research has predominantly been for determining the significance of the thiol group within the cysteine moiety of GSH (absent in ophthalmic acid) in the vast number of cellular processes that GSH is involved in. Coincidentally, it is also through these studies that potential roles of ophthalmic acid have been proposed. Such roles include its use as an anti-coenzyme that prevents the use of GSH as a co-factor by various detoxifying enzymes (Waley, 1953), a competitive inhibitor for the extracellular transport of GSH (Ballatori and Dutczak, 1994) and as a facilitator for the MRP1-mediated transport of glucuronide conjugates (Leslie *et al.*, 2001, Leslie *et al.*, 2003). All these functions, although requiring further investigation suggest that ophthalmic acid may act as a surrogate molecule for GSH during cellular stress where intracellular conservation of GSH may become an increasing priority. In a recent study where changes in the hepatic metabolome of mice treated with APAP were investigated, the level of ophthalmic acid was observed to increase during which GSH and cysteine were significantly decreased. It was therefore proposed that the decrease in cysteine which complemented the decrease in GSH may have triggered the increase in ophthalmic acid (Soga *et al.*, 2006) as a consequence of sharing the same synthetic machinery (Figure 1.8).

Ophthalmic acid is a tripeptide consisting of 2-aminobutyrate, glutamate and glycine. Its synthesis starts with the conjugation of 2-aminobutyrate and glutamate to form γ-glutamyl-2-aminobutyrate (γ-Glu-2AB) via the catalytic activity of GCL. This is then followed by the conjugation between γ-Glu-2AB and glycine to form ophthalmic acid, catalysed by GS (Figure 1.7) (Cliffe and Waley, 1958, Cliffe and Waley, 1961). As GSH and ophthalmic acid share the same enzyme for their synthesis (Figure 1.8), it has been suggested that levels in precursors, in particular cysteine, may influence the activities of these enzymes, which are either catalysing the synthesis of GSH, or ophthalmic acid. Through enzyme kinetic studies, GCL has been reported to have a greater affinity for cysteine than 2-AB suggesting that the GSH pathway is unsurprisingly, considering its multiple cellular functions, the more dominant pathway (Seelig and Meister, 1985). However, when cysteine levels are low, during oxidative stress for example, GCL activity may switch to the ophthalmic acid pathway. As such, ophthalmic acid has been proposed to be a potential biomarker of oxidative stress (Soga *et al.*, 2006).

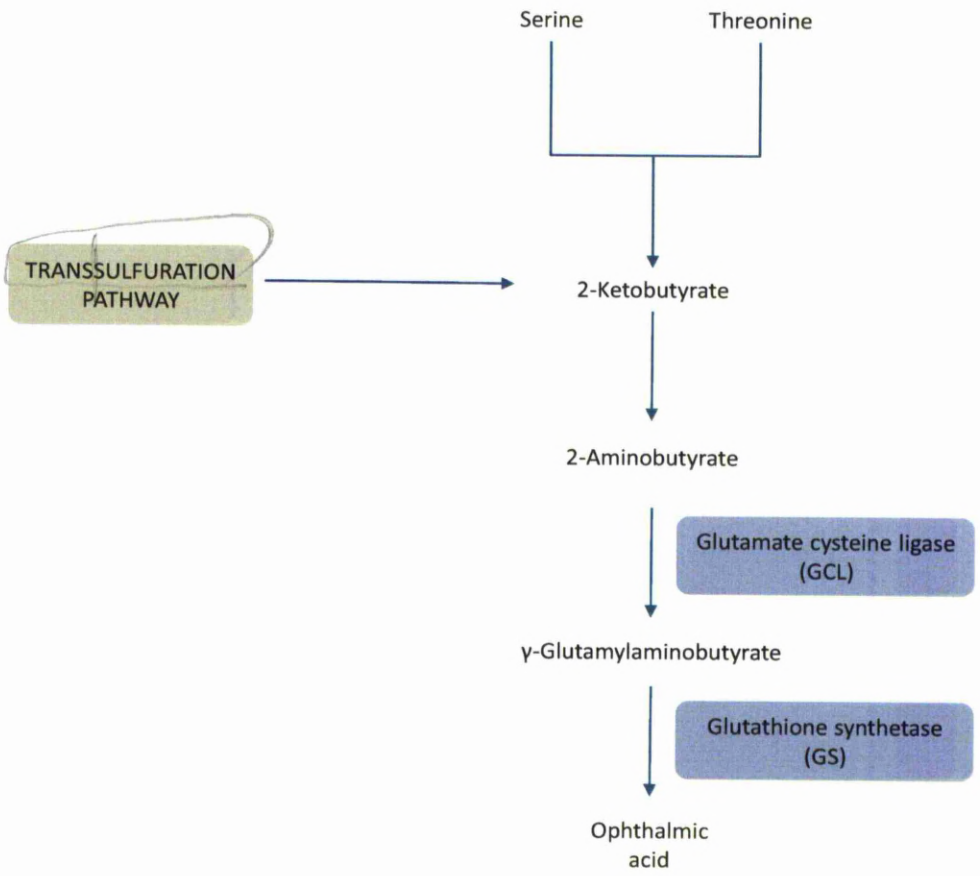


Figure 1.7 Ophthalmic acid synthetic pathway.

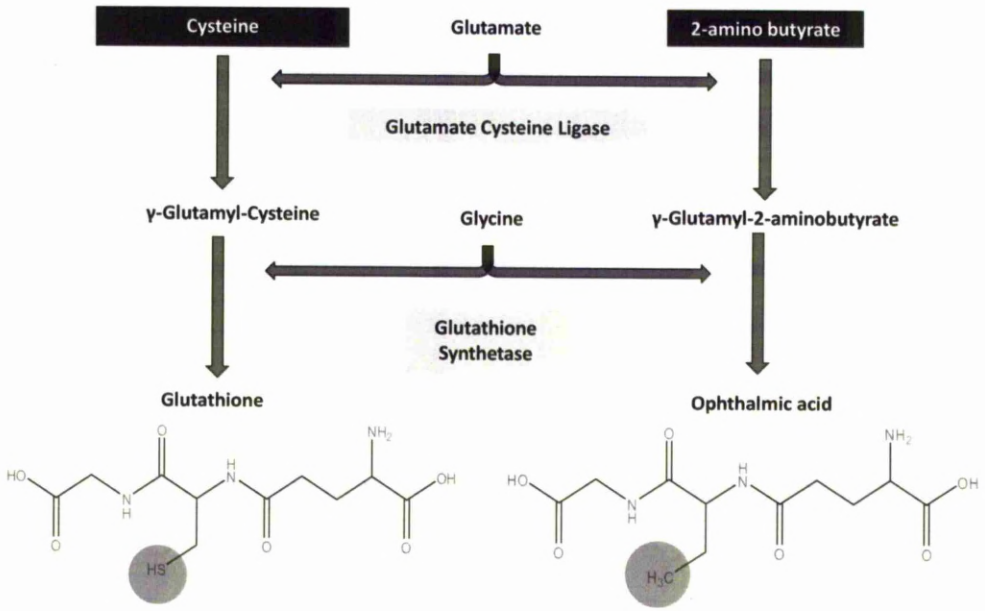


Figure 1.8 Similarities and differences between GSH and ophthalmic acid biosynthesis. GSH and ophthalmic acid use the same biosynthetic machinery for their synthesis, including the enzymes GCL and GS as well as the substrates glutamate and glycine. However, GSH and ophthalmic acid differ in in that in addition to glutamate and glycine, GSH synthesis also requires cysteine but in its place, 2-aminobutyrate is used during ophthalmic acid synthesis.

1.6.1.5 Detoxifying enzymes

In addition to non-protein antioxidants, phase I defence system also constitutes a number of enzymes that catalyse the deactivation of a variety of reactive species as summarised in Table 1.4.

Table 1.4 A summary of detoxifying enzymes and their respective activities in deactivating reactive species.

Enzyme	Function
Superoxide dismutase	Catalyses the formation of H_2O_2 from two molecules of superoxide ($O_2^{\cdot-}$). Particularly important in the detoxification of high levels of $O_2^{\cdot-}$ in the mitochondria during respiration.
Catalase	Catalyses the dismutation of H_2O_2 to water and O_2 using substrates such as phenols and alcohols.
Peroxiredoxin (Prx)	Directly reduces peroxides (H_2O_2 and lipid peroxides) and peroxynitrite. The active site thiol can be regenerated by thioredoxins (Trx), sulforedoxins and peroxiredoxins.
Glutathione Peroxidases (GPx)	Catalyses the reduction of H_2O_2 using GSH and other peroxides as substrates to alcohols.
Thioredoxins (Trx)	Specific donors of electron to many Prxs. Reduced Trx prevents apoptosis via an inhibitory binding to apoptosis signal-regulating kinase 1 (AKS-1) which is lost when Trx is oxidised.

1.6.2 Stage II: Up-regulation of antioxidants and detoxifying enzymes

When levels of cellular stress are enhanced and basal defence mechanisms can no longer detoxify reactive species sufficiently, an adaptive response is employed by the cell in which transcriptional up-regulation of cytoprotective genes is induced. This response is predominantly mediated by transcription factors, proteins that recognise and bind to certain DNA sequences upstream of the genes concerned, and in doing so, recruit both transcriptional and translational machineries for the expression of target genes. Key mediators include nuclear factor κB (NF-κB), activator protein 1 (AP-1), hypoxia-inducible factor 1 (HIF-1), aryl hydrocarbon receptor (Ah receptor or AHR) and nuclear factor

erythroid 2 (NF-E2)-related factor 2 (Nrf2), all of which are summarised in Table 1.5 except for Nrf2 which is discussed in more detail in section 1.6.2.1.

Table 1.5 A summary of transcription factors involved in the up-regulation of cytoprotective genes.

Transcription factor	Binding/recognition site	Stimuli for induction	Target genes
Nuclear factor-κB (NF-κB)	κB elements	Bacterial and viral infections, oxidative and endoplasmic reticulum stress, proinflammatory cytokines (TNFα, IL-10, IL-1B interleukin 1-B and chemical agents	Cyclooxygenase 2 (COX2), inducible intrinsic oxide synthase (iNOS), GST subunit P1-1 (GSTP1-1), GCL, SOD, p53, Bcl-xL and FasL.
Activating protein-1 (AP-1)	12-o-tetradecanoate-13-acetate (TPA) – responsive element (TRE) or Cyclic adenosine monophosphate (cAMP) – responsive element (CRE)	Bacterial and viral infection, cytokines, cellular and chemical stress.	GSTs, GCL, COX2, iNOS and various apoptotic regulators.
Hypoxia-inducible factor 1 (HIF-1)	Hypoxia		Growth factors
Heat shock factors (HSF)	High temperature		Heat-shock proteins
Aryl hydrocarbon receptor (Ah receptor or AHR)	Dioxin response element (DRE)	Ligan mediated. Chlorinated dioxins, related halogenated aromatic hydrocarbons, dibenzofurans, 2, 3, 7, 8-tetrachlorodibenzo-para-dioxin (TCDD) (Bradshaw *et al.*, 2002, Barouki *et al.*, 2007, Peng *et al.*, 2009).	CYP450 enzymes (Hillegass *et al.*, 2006), constitutive androstance receptor (CAR) (Patel *et al.*, 2007). For other target genes, refer to (Hankinson, 1995, Nebert *et al.*, 2000, Petrulis and Perdew, 2002).

1.6.2.1 Regulation by Nrf2 in cell defence

The role of Nrf2 in cell defence was first demonstrated by Itoh and colleagues (1997) in the Nrf2 null mice wherein these animals were observed to have a decreased ability in maintaining and up-regulating levels of a wide array of cytoprotective genes. This was further explored by various groups and have also reported this effect in a variety of tissues including the liver (Itoh *et al.*, 1997, Chan and Kwong, 2000, Kwak *et al.*, 2001, Ramos-Gomez *et al.*, 2001, Chanas *et al.*, 2002, Rangasamy *et al.*, 2005), lung (Chan and Kan, 1999, Cho *et al.*, 2002, Ishii *et al.*, 2005, Rangasamy *et al.*, 2005), skin (Xu *et al.*, 2006), brain (Lee *et al.*, 2003, Shih *et al.*, 2005, Kraft *et al.*, 2006) and the gastrointestinal tract (Itoh *et al.*, 1997, McMahon *et al.*, 2001, Khor *et al.*, 2006). Furthermore, these animals were also observed to demonstrate an enhanced susceptibility to xenobiotic and environmentally-induced stresses, one of the first examples having reported by Itoh and colleagues with butylhydroxyanisole (BHA) which resulted in a significant increase in the mRNA levels of NAD(P)H:quinone oxidoreductase (NQO1) and glutathione *S*-transferases (GST) in the Nrf2 wild type animals but not in their knock out counterparts. This effect was also demonstrated by various groups using a variety of chemical stress inducers including APAP (Chan *et al.*, 2001, Enomoto *et al.*, 2001), pentachlorophenol (Umemura *et al.*, 2006), carbon tetrachloride (Xu *et al.*, 2008) and arsenic (Jiang *et al.*, 2009). Common in the promoter region of these Nrf2 target genes is a consensus sequence (5'GAGTCACAGTGAGTCGGCAAAAATT-3') known as the antioxidant response element (ARE) that is recognised and bound to by Nrf2 along with a small musculo-aponeurotic factor (Maf) protein which in turn inititates gene transcription (Itoh *et al.*, 1997, Nguyen *et al.*, 2009).

Independently, investigations carried out by Reisman and colleagues on the therapeutic mechanism of oleanolic acid against liver injury also demonstrated the protective role of Nrf2 (Reisman *et al.*, 2009a). Oleanolic acid pre-treatment has been demonstrated to protect against a wide array of hepatotoxicants including acetaminophen, carbon tetrachloride (CCl_4), furosemide, bromobenzene and cadmium chloride (Liu *et al.*, 1993a, Liu *et al.*, 1993b, Liu *et al.*, 1994a, Liu *et al.*, 1994b, Klaassen and Reisman, 2010). Furthermore, oleanolic acid has also been shown to protect from various diseases including anaphylactic shock (Zhang and Ma, 1995) and diabetes (Gao *et al.*, 2009). However, it was only recently through work with paracetamol that Reisman and colleagues were able to demonstrate the ability of oleanolic acid to activate Nrf2 (Reisman *et al.*, 2009a).

Since the discovery of the protective role of Nrf2, cumulative research from various groups revealed Nrf2 as a master regulator of a wide array of cytoprotective genes including NAD(P)H:quinone oxidoreductase or NQO1 (Li and Jaiswal, 1992, Itoh *et al.*, 1997, Kwak *et al.*, 2001, McMahon *et al.*, 2001), glutathione *S*-transferases or GST (Itoh *et al.*, 1997, Knight *et al.*, 2008, Reisman *et al.*, 2009d), glutamate-cysteine ligase or GCL (Moinova and Mulcahy, 1999, Reisman *et al.*, 2009b), haem oxygenase-1 or HO-1 (Alam *et al.*, 1999) and multidrug resistance-associated proteins or MRPs (Maher *et al.*, 2005, Maher *et al.*, 2007). Furthermore, its protective role has also been implicated in various diseases such as cancer, inflammatory, neurodegenerative and cardiovascular diseases (Cho *et al.*, 2004, Cullinan *et al.*, 2004, Yu and Kensler, 2005, Leonard *et al.*, 2006, Kensler *et al.*, 2007, Johnson *et al.*, 2008, Yoon *et al.*, 2008, Kanninen *et al.*, 2009, Reddy *et al.*, 2009, Zakkar *et al.*, 2009, Boutten *et al.*, 2010, Jiang *et al.*, 2010, Cheng *et al.*, 2011) which as a consequence, has been exploited in the development of therapeutic interventions.

Although the protective role of Nrf2 has been well established, the mechanism(s) that regulate its activity is not, as is the topic of discussion in a paper published by Nguyen and colleagues (Nguyen *et al.*, 2009). However, one of these proposed mechanisms has to date been widely utilised and certainly exploited in the experimental work of many groups including that of our department and will be described in further detail below. Also, this particular mechanism will be referred to in the regulation by Nrf2 in cell defence.

One proposed mechanism in which Nrf2 activity is regulated is via its interaction with the cysteine-rich cytosolic protein, Kelch-like ECH-associated protein also known as Keap 1 as illustrated in Figure 1.9 (Itoh *et al.*, 1999, Dhakshinamoorthy and Jaiswal, 2001). In the absence of cellular stress, Keap1 plays a dual role, as a repressor by anchoring Nrf2 in the cytosol and as a facilitator of Nrf2 degradation (Itoh *et al.*, 1999, Dhakshinamoorthy and Jaiswal, 2001). The latter fate of Nrf2 is mediated by Cullin 3 (Cul3), an E3-based ligase that targets Nrf2 for ubiquitination and subsequent proteasomal degradation (Kobayashi *et al.*, 2004a). Consequently, this highly efficient and spontaneous degradation mechanism limits Nrf2 to a half-life of between 10 - 30 minutes (Furukawa *et al.*, 1987). Nrf2 up-regulation requires oxidative and/or electrophilic stimuli, resulting in the disruption of the Nrf2/Keap1 interaction (Itoh *et al.*, 1999) . This abolishes the ability of Keap1 to bind to newly synthesised Nrf2 allowing its translocation into the nucleus. Nuclear Nrf2 requires heterodimerisation with small Maf proteins in order to bind to the antioxidant response element (ARE), a DNA sequence motif (5'GAGTCACAGTGAGTCGGCAAAAATT-3') that is conserved amongst Nrf2 target genes (Itoh *et al.*, 1997). Nrf2, which is a member of the cap

'n' collar (CNC) family of transcription factors (Itoh *et al.*, 1995), is by far the most potent activator of the ARE (Kobayashi *et al.*, 1999). The binding of the Nrf2/Maf heterodimer to the ARE leads to the recruitment of transcriptional co-activators including the cAMP responsive element protein (CREB)-binding protein or CBP (Katoh *et al.*, 2001) which eventually initiates the transcription of a large battery of Nrf2-regulated genes.

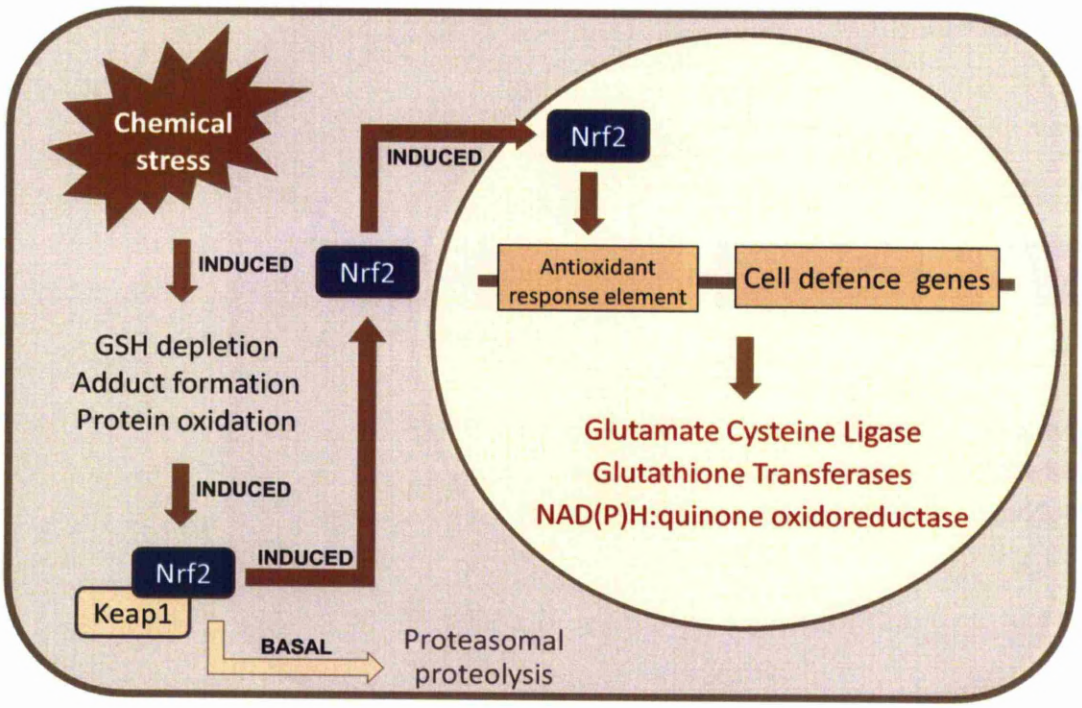


Figure 1.9 Regulation of Nrf2 activity. A schematic diagram describing the regulation of Nrf2 activity basally and upon induction by an increase in chemical stress as described in section 1.6.2.1.

1.6.3 Stage III: Programmed Cell Death

When phase I and phase II levels of cell defence become insufficient in protecting against extreme levels of cellular stress, the cell may resolve to the induction of programmed cell death through apoptosis for the containment of damage therefore preventing the spread of damage to surrounding healthy cells. Hallmarks of apoptosis include membrane blebbing, cell shrinkage, chromatin condensation and DNA fragmentation (Robertson and Orrenius, 2000).

1.7 BIOMARKERS

In addition to model systems that are used to investigate ADRs, biomarkers are also invaluable tools for use both in research and in the clinic to indicate the therapeutic threshold of drugs, which in turn could influence the application of drugs already in the market and in deciding whether those currently in the pipeline should be withdrawn.

A biomarker is a measurable indicator of either a natural biological process, a pathogenic process or a pharmacological process in response to a therapeutic intervention (Biomarkers Definitions Group, 2001). It is therefore a highly useful tool in a wide array of research and clinical applications including DILI. Ideally, a biomarker should be organ specific, sensitive for a particular type of injury, transferable across species, have low inter- and intra-individual variability, biologically relevant, present in biological samples that can be accessed in a non-invasive manner, stable, reduced when the insult or injury is resolved and amenable to testing. Years of research have led to the discovery of many biomarkers of DILI, however none of these have successfully fulfilled all the criteria outlined above.

1.7.1 Biomarkers in DILI

Biomarkers which are commonly used both in pre-clinical and clinical research and in the clinic to detect DILI include alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP) and total plasma bilirubin, all of which are endorsed by the US Food and Drug Administration (FDA). In particular, ALT and AST are more commonly referred to as the gold standard markers of hepatotoxicity. ALT and AST are leakage products of liver injury that are deposited into the blood. ALT displays higher sensitivity than AST and although both suffer from a lack of specificity, the more widespread expression of AST in extrahepatic tissues compared with ALT makes it a less specific marker (Ozer *et al.*, 2008, Yang *et al.*, 2009). ALT elevations may also prove to be false-positive (Edgar *et al.*, 1998, Thulin *et al.*, 2008) or false-negative (Solter *et al.*, 1998, Solter *et al.*, 2000). However, the problem with regards to the specificity of ALT could be resolved when taking into consideration the two isoenzymes, ALT1 and ALT2, each of which have different tissue specificity during DILI. However, at present, this approach could not be readily adopted as current commercially available and validated tests do not have the ability to differentiate between the two isoenzymes. Indeed, work is already underway in the development of a more sensitive and robust assay that will allow the specific detection and

quantification of these isoenzymes. Cytosolic AST and mitochondrial (mAST) have also been identified as two isoenzymes of AST that may add further specificity as a marker of liver injury, in particular for mAST, as a specific marker of alcohol-induced liver injury (Macchia *et al.*, 1997), however, further work is still required. ALP when used in conjunction with other markers such as ALT can be used as a specific marker of cholestatic DILI (Ramachandran and Kakar, 2009) however, ALP elevations may also be observed in other conditions such as bone disease and pregnancy (Reust and Hall, 2001) therefore its utility as a DILI biomarker is highly dependent on its use as a combination with ALT or AST. The liver is responsible for the clearance of bilirubin from the plasma. As a direct indicator of liver function (Senior, 2009), total plasma bilirubin is a more specific marker of liver injury in comparison to ALT, AST and ALP. However this advantage is still hampered by the ability of other conditions (Fabris *et al.*, 2009) to trigger elevations in total plasma bilirubin therefore measurement of other parameters is still required.

Although a common practice has now been adopted where several markers are measured during the study of DILI, continued effort is still made by various groups in search for biomarkers that may offer better sensitivity and specificity compared with the current standards. At present, several promising biomarkers have been reported that offer better sensitivity and specificity for predicting liver injury, although may still require confirmation with other biomarkers. A summary of potential markers of DILI that are currently under investigation is presented in Table 1.6.

Table 1.6 A summary of potential markers of DILI currently under investigation. Adapted from Ozer (2008) and Antoine *et al.*, (2009). References for each biomarker are available in Appendix 5.

Biomarker	Function	Tissue localisation	Damage	Compartment
Malate dehydrogenase (MDH)	Catalyses the reversible transformation of malate to oxaloacetate in the Kreb's cycle.	Liver, hear, skeletal muscle, brain	Mitochondrial damage Necrosis	Blood
α-glutathione-S-transferase (GST-α)	Phase II detoxifying enzymes that catalyse the conjugation of GSH with reactive metabolites formed.	Liver, kidney	Necrosis	Blood
Purine nucleotide phosphorylase (PNP)	Catalyses the phosphorolysis of nucleosides to their respective bases and corresponding 1-(deoxy)-ribose-phosphate.	Liver, heart, muscle Localised in endothelial cells, Kupffer cells and hepatocytes.	Necrosis (leakage)	Blood
Paraoxonase 1 (PON-1)	A high-density lipoprotein *HDL)-associated esterase. Detoxifies organiphosphates in the liver. Protects low-density lipoproteins from oxidative modifications.	Liver, kidney, brain and lung	Liver function – released into circulation bound to HDL. Necrosis	Blood
Arginase 1	A hydrolase that catalyses the catabolism of arginine to urea and ornithine.	Liver	Necrosis (leakage)	Blood
Serum F protein (4-hydroxyphenylpyruvate dioxygenase (HPD))	Key enzyme in tyrosine metabolism.	Liver and kidney	Necrosis (leakage)	Blood
Cytochrome c	A small heme protein that is essential in ATP generation and functions as an electron shuttle between complexes II and IV of the electron transport chain. It is also plays an important role in mitochondria-mediated apoptosis.	Liver, kidney, skeletal muscle and hear	Mitochondrial damage	Blood/urine
High mobility group box protein 1 (HMGB1)	Nuclear binding protein that has pro-inflammatory activity and targets toll-like receptors (TLR) and the receptor for advanced glyucation end-products (RAGE) on target cells.	Liver	Necrosis (not apoptosis) and inflammation	Blood
Ophthalmic acid	Biological function has not yet been established. However, proposed functions include, inhibitor of enzymes that use GSH as a co-factor, competitive inhibitor and trans-stimulator of GSH uptake and substitute for active transport of glucuronide metabolites by MRP-1	Lens, kidney, spleen, liver and brain	Oxidative stress	Blood
Keratin-18 (K18) - fragmented	Intermediate filament proteins responsible for cell structure and integrity.	Liver	Apoptosis, not necrosis	Blood
Keratin-18 (K18) – full length	Intermediate filament proteins responsible for cell structure and integrity.	Liver	Necrosis, not apoptosis	Blood

1.7.2 Ophthalmic acid as a potential biomarker of chemical stress

Hepatic GSH depletion is a common feature of DILI and as such is routinely used as a marker of chemical stress in pre-clinical studies. However, its use is limited to pre-clinical applications as it is rapidly degraded in the blood by γ-GGT therefore analysis in humans is a challenge. Recently, as described earlier in this chapter, ophthalmic acid has been proposed as a potential surrogate marker of GSH depletion (Soga *et al.*, 2006). Although GSH depletion is not necessarily a marker of hepatotoxicity, an alternative marker that reflects GSH status in addition to the current panel of markers could contribute to the level of sensitivity and to some extent, specificity, in the prediction of DILI. Indeed, drugs that have the potential to cause hepatotoxicity exert this ability through different modes, one of which could be oxidative stress. GSH depletion would therefore play a significant role considering its importance in maintaining the cell's redox status as discussed in section 1.6.1.2. A biomarker of GSH depletion may therefore aid in elucidating the hepatotoxic potential of drugs. Further investigation into the potential of ophthalmic acid as a marker of GSH consumption is required as very little work on its biological function has been undertaken so far. Furthermore, Soga and colleagues (2006) suggested that the increase in ophthalmic acid in APAP-treated mice was due to a combination of cysteine and GSH depletion. Determination of the exact trigger of ophthalmic acid increase therefore needs to be elucidated as the depletion in GSH observed may just be a consequence of cysteine depletion, cysteine being a precursor of GSH which contains the thiol group that is essential for the wide array of biological activity in which GSH is involved in.

1.8 AIMS OF THE PROJECT

In light of the problem still posed by ADRs, despite the vast body of knowledge that is available on the toxic mechanisms of ADRs as well as the defence systems deployed to combat it, the aim of this thesis was to develop novel model systems to allow investigations in the mechanisms of DILI and to facilitate the development of novel biomarkers of drug-induced chemical stress. In particular, the human liver epithelial cell line, THLE-2E1, which has been engineered to overexpress the CYP2E1 enzyme, the $Nrf2^{(-/-)}$ mouse and the endogenous metabolite ophthalmic acid were assessed for their potential as models of chemical stress.

The THLE-2E1 cell line was assessed to determine its utility as a model of oxidative stress in the absence or upon induction of a metabolic substrate. The THLE-2E1 cell line

would be an ideal model for investigating oxidative stress as overexpression of the CYP2E1 enzyme has been shown to be pro-oxidant due to the incomplete coupling between this enzyme and NADPH-cytochrome P450 reductase. The $Nrf2^{(-/-)}$ mouse was investigated for its potential use in determining the extent of the role of Nrf2 in DILI, in particular, the extent of its regulatory role in cellular defence. Work carried out in this model specifically looked at the role of Nrf2 in the regulation of the GSH system. Finally, ophthalmic acid was investigated as a biomarker of oxidative stress as it has been demonstrated to significantly increase upon depletion of hepatic GSH as a result of APAP treatment. As described in section 1.6.1.2 and discussed further in chapter 3, GSH plays a central role in cellular defence in addition to other physiological processes within the cell. A biomarker that could reflect the behaviour of GSH during a toxic insult is therefore valuable in determining the susceptibility of a cell to toxicity in response to a particular drug treatment. As such, as part of the work carried out on ophthalmic acid, perturbations right across the GSH system were also investigated.

Chapter 2

Evaluation of the THLE-2E1 cell line as an *in vitro* model of chemical stress

TABLE OF CONTENTS

2.1 INTRODUCTION

The use of primary human hepatocytes continues to be the preferred *in vitro* route for determining and extrapolating the metabolic and toxicity profiles of a drug to a clinical setting (Guillouzo *et al.*, 1993, LeCluyse, 2001, Bjornsson *et al.*, 2003, Gomez-Lechon *et al.*, 2004). However, such systems have a limited life-span, are highly variable with regards to cytochrome P450 (CYP450) contents and activity and dedifferentiate rapidly following isolation (Bjornsson *et al.* 2003; Cross *et al.* 2000; Guillouzo *et al.* 1993). It is also difficult to access a consistent supply of freshly isolated cells, which are preferable to the cryopreserved alternatives. Consequently, many groups have opted for using immortalised human cell lines as an alternative as they have proven easier to maintain, are long-lived and readily available. One major disadvantage however, is the absence of metabolic activity as a consequence of transformation (Rowe *et al.*, 2010). In order to address this deficit, cell lines have been developed which are engineered to overexpress individual CYP450 isoforms constitutively or through the use of an inducible promoter (Dai *et al.*, 1993, Chen and Cederbaum, 1998, Goldring *et al.*, 2006).

Recently, a panel of SV40 T-antigen-immortalised human liver epithelial cells (THLE) has been stably transfected with specific CYP450 isoforms (CYP-1A2, 2C9, 2D6, 2E1 or 3A4), with expression transcriptionally driven by a cytomegalovirus (CMV) promoter and selected for by the G418 selective gene (Pfeifer *et al.*, 1993, Mace *et al.*, 1997). These cells were derived from the THLE-5B parental immortalised human hepatocyte cell line which exhibits hepatocyte-like phenotype, with high levels of reduced GSH and phase II enzyme activities except for UGTs (Pfeifer *et al.*, 1993, Dambach *et al.*, 2005). In conjunction with work within the Molecular Toxicology group at AstraZeneca (Alderley Park, UK), all THLE-CYP cell lines were characterised and assessed for their utility in drug toxicity studies. The focus of this chapter was to characterise and evaluate the THLE-2E1 cell line for its potential as an *in vitro* tool for studying chemical stress.

The significance of a CYP2E1 cell line for the study of chemical stress stems from the intrinsic ability of this CYP450 isoform to elicit oxidative stress in the absence of an inducer. Compared with other CYP450 isoforms, CYP2E1 has the ability to constitutively produce high levels of ROS (Gorsky *et al.*, 1984, Dai *et al.*, 1993) due to its weak coupling with CPR as demonstrated in isolated microsomes and reconstituted enzyme systems (Gorsky *et al.*, 1984, Ekstrom and Ingelman-Sundberg, 1989). This unique feature of CYP2E1

was also reported in a human hepatocarcinoma cell line developed to overexpress this enzyme (HepG2-2E1) (Dai *et al.*, 1993). The authors of this study associated the increased level of oxidative stress observed in these cells with the up-regulation of glutathione (GSH) synthesis (Mari and Cederbaum, 2000).

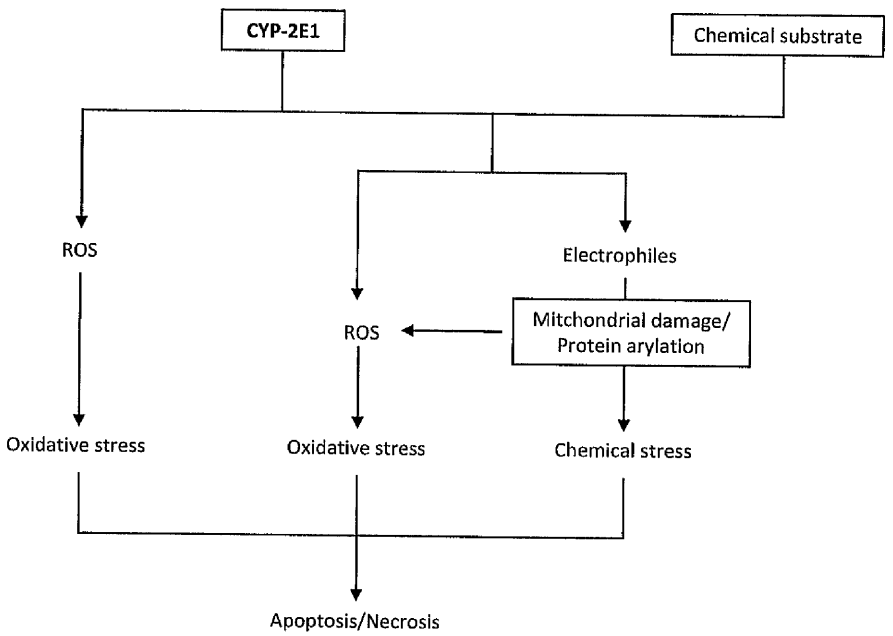


Figure 2.1 A schematic diagram summarising the pathways by which CYP2E1 elicits cellular stress. CYP2E1 exhibits enhanced NADPH oxidase activity compared with other CYP450 isoforms due to its poor coupling with CPR, resulting in the constitutive production of free radicals/ROS including superoxide anion ($O2^{\cdot-}$), H_2O_2 and hydroxyl radicals ($^{\cdot}OH$), all of which lead to oxidative stress (Gorsky *et al.*, 1984, Ekstrom and Ingelman-Sundberg, 1989). This can be further potentiated through CYP2E1-mediated metabolism of a chemical substrate such as APAP, which can be bioactivated into the chemically reactive metabolite (electrophile) NAPQI (Raucy *et al.*, 1989). Cellular damage such as that in the mitochondria and protein arylation caused by both ROS and electrophiles that can no longer be managed by the cell's defence system may lead to the dysfunction of various cellular processes, potentially resulting in cell death (apoptosis/necrosis).

The ability of CYP2E1 to produce a state of oxidative stress can be potentiated through the addition of chemical substrates. In particular, the CYP2E1-mediated toxicity elicited by ethanol (Morimoto *et al.*, 1994, Nanji *et al.*, 1994, Lieber, 1999, Bradford *et al.*, 2005, Gonzalez, 2005, Cederbaum, 2006) and APAP (Mitchell *et al.*, 1973b, McClain *et al.*, 1980, Morgan *et al.*, 1983, Raucy *et al.*, 1989, Lee *et al.*, 1996, Zaher *et al.*, 1998, Manyike *et al.*, 2000, Cheung *et al.*, 2005) are well documented and continue to have a significant impact on public health. Furthermore, CYP2E1-mediated metabolism has also been

implicated in the carcinogenicity of certain compounds such as nitrosamines and azocompounds (Yang *et al.*, 1990, Guengerich *et al.*, 1991, Sohn *et al.*, 1991, Sohn *et al.*, 2001). As APAP is a well documented substrate for CYP2E1 bioactivation to the electrophile NAPQI (chapter 1 section 1.5.1), where at overwhelming levels consequently causes toxicity to the human population even to this day (Ostapowicz *et al.*, 2002), APAP has been chosen as the chemical model for evaluating the potential of THLE-2E1 as an *in vitro* model of chemical stress.

Although CYP2E1 overexpressing cells have been previously developed and are in current use (Dai *et al.*, 1993, Chen and Cederbaum, 1998, Goldring *et al.*, 2006), the THLE-2E1 cell line is unique for being a member of a panel of CYP overexpressing cells that originate from one parental immortalised cell line. Furthermore, this panel of cell lines also include the THLE-Nulls which have been transfected with an empty vector, providing a direct control during studies that utilises the CYP overexpressing cells, such as those described in this chapter. As inter- and intra-species variability in the metabolic and toxicity profiles of drugs is a common problem (Turpeinen *et al.*, 2007), the availability of the THLE-CYP cells could potentially provide the capability to elucidate the metabolic and toxic profiles of a drug candidate within a specific model system at the pre-clinical stage. As such, the aims of the work described in this chapter were:

1. To characterise the THLE-2E1 cell line which have not been previously assessed in terms of viability, growth characteristics, CYP2E1 expression levels and metabolic capacity using the CYP2E1 model substrate chlorzoxazone.
2. To assess the THLE-2E1 cells as a model system for exploring oxidative stress in the absence of metabolic substrates using the THLE-Null cells as a comparator.
3. To assess the THLE-2E1 cells' capacity for the metabolic activation of a known CYP2E1 substrate (APAP) as a model of oxidative stress.

2.2 MATERIALS AND METHODS

2.2.1 Materials

All chemicals and media supplements were purchased from Sigma-Aldrich (Poole, Dorset, UK) and Invitrogen (Paisley, UK) unless otherwise stated. Cell culture media (Basal PMFR-4) was purchased from Millipore (Billerica, Massachusetts, USA). Sterile Biocoat™ cell culture flasks and plates were purchased from Becton Dickinson (Oxford, UK). Nunclon Δ cell culture dishes were from Nalge-Nunc International (c/o VWR International, Lutterworth, Leicestershire, UK). All other plasticware were sourced from Fisher (Loughborough, Leicestershire, UK). CellTiter 96 AQ_{ueous} Non-Radioactive Cell Proliferation Assay (MTS assay) was purchased from Promega (Southampton, Hampshire, UK). Precision Plus Protein Kaleidoscope standards and non-fat dry milk were from Bio-Rad (Hemel Hempstead, Hertfordshire, UK). RIPA buffer and Protease Cocktail Inhibitor were purchased from Thermo Scientific (Basingstoke, Hampshire).

THLE-CYP cells (Null and CYP2E1) were obtained under license from Nestec Ltd, Switzerland. Briefly, the non-tumourigenic human liver epithelial cell line 5B (THLE-5B) was immortalised by transfection with a simian virus 40 large T antigen. The THLE-5B was used as the parental cell line for establishing the THLE-CYP cell line panel by transfection with individual cytochrome P450 cDNA (including CYP2E1 and an empty vector for THLE-Null) driven by a cytomegalovirus (CMV) promoter (Figure 2.2) (Pfeifer *et al.*, 1993). The THLE-CYP cells have been demsontrated to retain characteristics of the parental THLE-5B cells, all of which express both phase I and phase II enzymes with the exception of UGTs (Mace *et al.*, 1997).

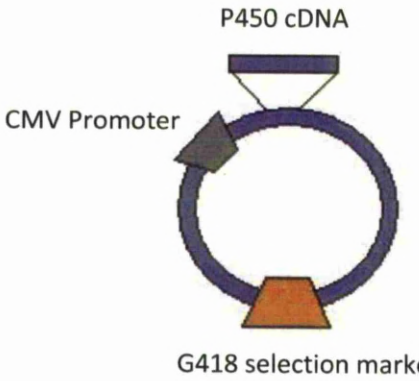


Figure 2.2 A generalised plasmid construct used to transfect the THLE-5B cells into specific THLE-CYP and THLE-Null cell lines. The THLE-Null cell line was established using a plasmid construct that doesn't contain a CYP450 cDNA. The high expression of each CYP450 isoform is driven by a CMV promoter and maintained and selected for by the G418 resistance gene (Pfeifer *et al.*, 1993).

2.2.2 Cell culture

2.2.2.1 Media preparation

THLE-CYP cells were maintained in complete media (CM) which comprised Basal PMFR-4 media supplemented with 2 mM GlutaMAX, 1.75 µM insulin transferrin selenium (ITS), 1 nM hydrocortisone, 0.5 ng/ml epidermal growth factor (EGF, reconstituted at 0.1 mg/ml in 10 mM acetic acid/0.1 % bovine serum albumin (BSA)), 35 µg/ml bovine pituitary extract (BPE), 0.33 nM retinoic acid, 3 % fetal bovine serum (FBS) and 150 µg/ml G418. Serum free media (SFM) which has the same composition as CM less ITS and FBS was used for drug incubations.

2.2.2.2 Growing of cryopreserved cells

Cryopreserved cells (1 x 10^6 cells/ml and prepared as detailed below in section 2.2.2.5) were subjected to a quick thaw step in a pre-heated 37 °C water bath and immediately transferred into a sterile Biocoat ™ T75 flask with 15 ml complete media (CM). The cells

were allowed to attach to the surface of the flask for a minimum of 4 hours (h) in a humidified chamber at 37 °C under 5 % CO_2. This was followed by the removal of the media and replacement with a further 15 ml fresh CM for cell growth. Media was routinely replaced every two days until growth reached 80 % confluency at which point the cells were passaged. Cells used were between passages 13 - 20.

2.2.2.3 Cell passaging

Passaging of the cells involved a wash step with Hank's balanced salt solution (HBSS, -Ca^{2+}/-Mg^{2+}), followed by treatment with 0.025 % trypsin-EDTA (1 ml) for 3 minutes at room temperature to allow the detachment of cells from the surface. Further trypsinisation was prevented by the addition of 10 ml CM which was also used to collect the detached cells in a suspension.

2.2.2.4 Cell counting

A mixture of cell suspension as acquired in section 2.2.2.3 and 0.2 % Trypan Blue at a 1:1 ratio was prepared. Using a haemocytometer (Mods-Fuchs Rosenthal BS748 depth 0.2 mm, 0.0625 mm^2), 20 µl of this mixture was counted to calculate the number of cells/ml using the equation below:

$$\text{Cells/ml} = ((a + b + c + d)/4) \times CF \times DF$$

Where CF is the Conversion Factor = 5000, DF is the Dilution Factor = 2 and a, b, c and d are the number of cells per square on the haemocytometer.

2.2.2.5 Cryopreservation of cells

Cells were harvested as detailed in section 2.2.2.3 and collected in a pellet by centrifugation at 1200 revolutions per minute (rpm) for 3 minutes at room temperature. The media was carefully aspirated and the cell pellet reconstituted in freezing media (1:1 ratio of Basal PMFR-4 media (without supplements):FBS containing 10 % DMSO). The cells were counted as detailed in section 2.2.2.4 and divided into sterile Nunc cryopreservation tubes at 1 x 10^6 cells/ml. The cells were slowly frozen down in an isopropanol containing cryopreservation

chamber at -80 °C for 24 h and were subsequently transferred into a liquid nitrogen chamber for long term storage.

2.2.3 Cell morphology

Comparison of cell morphology was carried out by microscopy (Leica DM IRB microscope and Leica CD 200 camera; Leica Microsystems, Milton Keynes, Buckinghamshire, UK) on a 24 h culture of both THLE-Null and THLE-2E1 cell lines in CM.

2.2.4 Cell growth rate

THLE-Null or THLE-2E1 cells were seeded at 250 x 10^3 cells/well in a Biocoat ™ T25 flask and left to grow in 5 ml CM for 72 h. Media was replaced for both cell lines as and when required within the allocated growth period. Cells were harvested and counted as detailed in sections 2.2.2.3 and 2.2.2.4 and values were used to calculate the doubling time of each cell line.

2.2.5 Cell growth curve

Increasing number of THLE-Null and THLE-2E1 cells were seeded (1 x 10^3 – 40 x 10^3 cells/well) in Biocoat ™ 96 well plates for 24 h in triplicate. Mitochondrial activity as a measure of cell viability was determined as detailed below (section 2.2.6).

2.2.6 Mitochondrial activity determination

Mitochondrial activity was measured as an indicator of cell viability using a Promega CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay™ kit. MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) solution was diluted 1 in 5 with serum-free media (SFM). All treatment media in the incubation plates were removed and replaced with 120 µl of MTS solution. The reaction was allowed to take place in a humidified chamber at 37 °C under 5 % CO_2 for 1 h. MTS solution (100 µl) was removed from the incubation plate and transferred into a Costar 96-

well plate where the absorbance was immediately read using a Wallac™ EnVision® spectrophotometer (PerkinElmer; Seer Green, Buckinghamshire, UK) at 492 nm.

2.2.7 Protein content determination using the Bradford method

Protein concentration was measured based on the Bradford method (Bradford, 1976) using the Protein Assay Dye Reagent. The Bradford method relies on the binding of the Coomassie Brilliant Blue G-250 dye to basic and aromatic amino acids resulting in a colour change of the dye from red to blue with a resulting change in absorbance maximum from 465 to 595 nm. The protein content was determined by measuring the increase in absorbace at 570 nm using an MRX microplate reader (Chantilly, VA) which is proportional to the amount of bound dye and therefore the amount of protein present. A standard curve prepared from bovine serum albumin fraction V (0 – 2 mg/ml) was used to calculate the protein content.

2.2.8 Protein content determination using the Lowry method

For samples with low protein content, protein concentration was measured using the Lowry method (Lowry *et al.*, 1951). Samples and protein standards (1 mg/ml stock solution of bovine serum albumin fraction V in dH_2O diluted to concentrations between 0-0.2 mg/ml) were diluted as appropriate in 0.5 M NaOH. Prepared samples and standards were added with 50 µl Lowry Reagent (0.5ml 1% $CuSO_4.5H_2O$, 0.5ml 2% NaK Tartarte and 10ml 10% Na_2Co_3 in 0.5M NaOH) and allowed to incubate for 15 min at room temperature. This was followed by the addition of 150 µl Folins Reagent (diluted 1 in 10 with dH_2O) and samples and standards were left to react in the dark at room temperature for a further 30 min. The absorbance was measured on an MRX plate reader (Chantilly, VA) at 570 nm.

2.2.9 Determination of total GSH content

GSH content was measured according to the 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB)-GSH reductase recycling method (Vandeputte *et al.*, 1994). Briefly, proteins prepared in section 2.2.10 were pelleted by centrifugation at 14 000 rpm for 5 min. A 1:1 ratio (at 20 µl of each, v/v) of the supernatant and GSH buffer (0.143 M NaH_2PO_4, 6.3 mM EDTA, pH 7.4) were

combined in a Nunclon Δ 96-well plate to neutralise the pH. Samples were incubated with the assay reagent (200 µl; 1 mM DTNB, 0.34 mM NADPH, in 0.143 M NaH_2PO_4, 6.3 mM EDTA, pH 7.4) for 5 min at room temperature. The enzymatic reaction was initiated by the addition of 0.35 U GSH reductase and immediately read at 405 nm for 5 min using an MRX microplate reader (Chantilly, VA). GSH concentrations of the samples were calculated using a GSH standard curve ranging between 1 – 80 nmol/ml GSH. GSH content for each sample was normalised to total protein content measured as described in sections 2.2.7 and 2.2.8.

2.2.10 Preparation of protein extracts

THLE-Null and THLE-2E1 cells were seeded (250 x 10^3 cells/well) in a Biocoat ™ T25 flask in triplicate for 24 h in a humidified chamber at 37 °C under 5 % CO_2 and subsequently harvested as detailed in section 2.2.2.3. Cell suspensions were centrifuged at 1200 rpm for 3 minutes at 24 °C after which the media was carefully aspirated. The cell pellets were washed with HBSS (-Ca^{2+}/-Mg^{2+}) and collected by a further centrifugation step. Cytosolic extracts were obtained through cell lysis with 200 µl RIPA buffer supplemented with protease cocktail inhibitor. The resulting supernatant and pellet samples were stored at -80 °C until required. Determination of protein concentrations were performed as detailed in section 2.2.7 or 2.2.8.

2.2.11 Western blot

Whole cell lysates (30 µg) were mixed with 5µl NuPage Master Mix (3.5µl LDS sample buffer and 1.5µl reducing reagent). Proteins were denatured at 80 °C for 10 min. Samples were loaded onto pre-made 4-12% NuPage Novex Bis-Tris gels alongside full range molecular weight rainbow markers (Precision Plus Protein Kaleidoscope standards) and two cryopreserved human hepatocyte prepared as for the THLE cells (HL-hep) and a human liver homogenate (HL-hom) as positive controls. The samples were separated by denaturing electrophoresis using 3-(N-Morpholino) propapane sulfonic acid (MOPS)-SDS running buffer (1x) at 90 V for 10 min which was increased to 170 V for a further 1 h. The resolved proteins were transferred to nitrocellulose membrane for 1 h at 80 V using a 1x transfer buffer (glycine 50mM; Tris 25mM; 1l) with 20 % methanol (v/v). An ice pack was included in the transfer apparatus with a magnetic stirrer to ensure even distribution of heat.

Following protein transfer, the membrane was rinsed in 0.1 % Tween-Tris buffered saline (T-TBS; 150mM NaCl, 3mM KCl, 25mM Tris, 0.1 % Tween 20) and stained with Ponceau red dye to ensure equal protein loading and transfer of protein extracts. The membrane was again rinsed with 0.1 % T-TBS and blocked (30 min, 10 % blotting grade non-fat milk in 0.1 % T-TBS) to reduce non-specific binding. For specific CYP2E1 protein detection, the membrane was incubated with a rabbit anti-CYP2E1 IgG antibody (Abcam, Cambridge, UK); 1:3000 in 0.1% T-TBS containing 2 % milk; 10ml) for 1 h at room temperature. The membrane was repeatedly washed (4 x 5 min washes) with 0.1 % T-TBS and then further incubated with a secondary antibody, goat anti-rabbit (Sigma-Aldrich, Poole, Dorset, UK), 1:3000) for 1 h at room temperature. The membrane was again subjected to a multiple wash step. CYP2E1 protein-antibody conjugate was visualised using Western Lightning Plus chemiluminescence reagent (PerkinElmer) and membranes were exposed to ECL film (Amersham; GE Healthcare, Little Chalfont, Buckinghamshire, UK).

2.2.12 Coomassie stain

Proteins (section 2.2.10) were resolved as detailed in section 2.2.11. The resolved proteins were fixed in a solution of 7 % glacial acetic acid in 40 % (v/v) methanol for a minimum of 1 h and were subsequently stained with Brilliant Blue G Colloidal Coomassie dye (diluted 4:5 (v/v) with methanol) for 2 h. Excess Coomassie stain was removed by washing the gel with a solution of 10 % acetic acid in 25 % (v/v) methanol for 1 minute, followed by a further rinse step with 25 % methanol.

2.2.13 CYP2E1 activity

Chlorzoxazone was used as substrate for the determination of CYP2E1 activity (Figure 2.3). THLE-Null and THLE-2E1 were seeded at 250 x 10^3 cells/well on a Biocoat™ 6-well plate format overnight in a humidified chamber at 37 °C under 5 % CO_2. Cells were treated with increasing concentrations of chlorzoxazone (0 – 500 µM) in SFM for 2 h. The reaction was stopped with the addition of equal volumes of ice-cold acetonitrile to the drug solution (v/v) which were collected and transferred into tubes and stored at -80 °C until analysis.

Cell debris from the samples was removed by centrifugation (14 000 rpm, 4 °C, 10 min). Clean supernatants were transferred into new tubes and spiked with 1 µM

phenacetin (PNC) in 50 % MeOH. The samples were dried down by centrifugation under vacuum (SpeedVac, manufacturer, address) and subsequently reconstituted in 50 % MeOH (400 µl). Concentrated samples were analysed for 6-hydroxy chlorzoxazone by LC-MS-MS.

All LC-MS-MS experiments were performed using a Perkin Elmer SCIEX API 3000 LC-MS-MS system with a Perkin Elmer Series 200 Autosampler and HPLC Pump (Massachusetts, USA). Analytes were separated using a Silica Uptisphere C18 column 5 µ ODB, 100 x 2 mm length (Interchim c/o Cheshire Sciences Chester, UK). The flow-rate of 200 µL/min was achieved with an elution gradient composed of solvent A (0.1 % formic acid in dH_2O) and solvent B (100 % MeOH). The gradient started with a linear gradient from 100 – 85 % for 5 min, 85 – 40 % for 10 min, 40 – 100 % for 3 min and finished with an isocratic step at 100 % for 2 min with solvent A. The total analysis time and injection volume were 20 min and 50 µl respectively. The autosampler syringe was subjected to three pre and post sample injection washes with solvent A. Analytes were detected in the positive ion mode using MS-MS with multiple reaction monitoring (MRM) which uses the unique fragmentation pattern of each compound for detection and quantification. The source temperature was set at 350 °C and nebuliser gas set at 12. Collision energies and transition ion pairs were optimised for each analyte using reference compounds (Figure 2.4). System control and data acquisition were performed using Analyst Version 1.4 software.

Chlorzoxazone

6-OH Chlorzoxazone

Figure 2.3 CYP2E1-mediated metabolism of chlorzoxazone to 6-OH chlorzoxazone

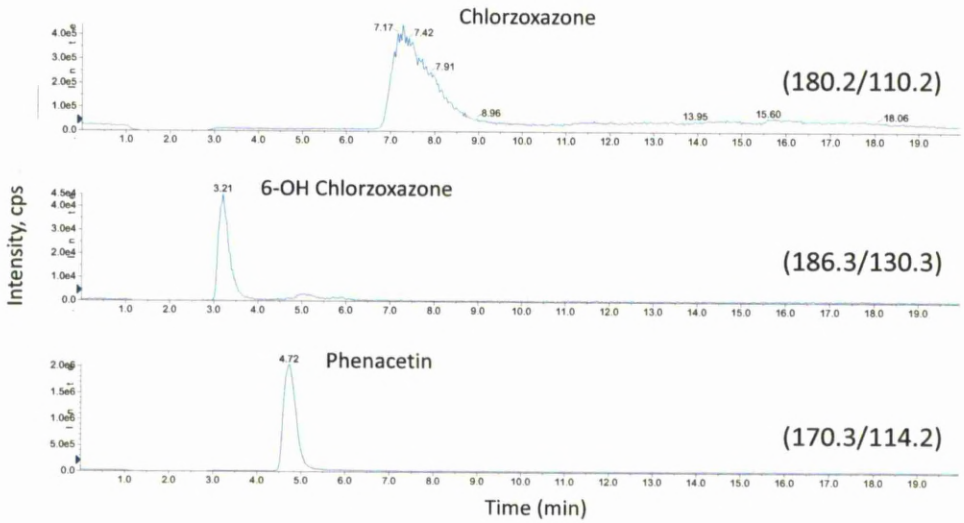


Figure 2.4 LC-MS-MS chromatograms of Chlorzoxazone, 6-OH Chlorzoxazone and Phenacetin. A mixture of chlorzoxazone, 6-OH chlorzoxazone and phenacetin (internal standard) was analysed by LC-MS-MS using the MRM technique (MRM transitions in brackets) (section 2.2.13). The same LC-MS-MS method was used to analyse CYP2E1 activity in the THLE-2E1 cells.

2.2.14 APAP metabolite identification

THLE-Null and THLE-2E1 cell lines were seeded at 250 x 10^3 cells/well in a Biocoat™ 6-well plate format for metabolite identification. Each cell line was treated with increasing concentrations of APAP (0 – 25 mM) prepared in SFM for 24, 48 and 72 h. After incubation, the media was collected and added with equal volumes of 100 % MeOH. Cell debris from the samples were removed by centrifugation (14 000 rpm, 4 °C, 10 min) and monitored for the APAP metabolites as described below.

All LC-MS-MS experiments performed in this section were carried out on instruments detailed in section 2.2.13. The flow-rate of 200 µL/min was achieved with an elution gradient composed of solvent A (0.1 % formic acid in dH_2O) and solvent B (100 % MeOH). The gradient started with a linear gradient from 100 – 96 % for 3 min, 96 – 20 % for 22 min, an isocratic step at 20 % for 5 min, a gradient step from 20 – 100 % and finally an isocratic step at 100 % for 1 min with solvent A. The total analysis time and injection volume were 41 min and 50 µl respectively. The autosampler syringe was subjected to three pre and post sample injection washes with solvent A. Analytes were detected in the positive ion mode using MS-MS with MRM which uses the unique fragmentation pattern of each compound for detection and quantification. The source temperature was set at 450 °C

and nebuliser gas set at 15. Transition ion pairs used for the glucuronide, cysteine, cysteinylglycine, glutathione and sulphate conjugates were 328>152, 271>140, 328>182, 457>328 and 232>152 respectively and adopted from (Kostrubsky *et al.*, 2005) which were validated by (Mutlib *et al.*, 2000). System control and data acquisition were performed using Analyst Version 1.4 software.

2.2.15 APAP toxicity

THLE-Null and THLE-2E1 cell lines were seeded at 15 x 10^3 cells/well in a BiocoatTM 96-well plate format for cytotoxicity analysis. Each cell line was treated with increasing concentrations of APAP (0 – 25 mM) prepared in SFM for 24, 48 and 72 h. APAP toxicity was assessed following the MTS assay detailed in section 2.2.6. GSH content was also measured following the protocol detailed in section 2.2.9.

2.2.16 Statistical analysis

Where applicable, all data were expressed as mean ± standard error of the mean (SEM). Values to be compared were analysed for normal or non-normal distribution using a Shapiro-Wilk test. Unpaired t-tests or one-way ANOVA were used when normality was indicated. A Mann-Whitney U or Kruskall-Wallis test was used when the data were determined to be non-normally distributed. All statistical tests were performed using StatsDirect and results were considered to be significant when $p < 0.05$.

2.3 RESULTS

In order to evaluate the utility of the THLE-2E1 cell line as an *in vitro* tool for investigating chemical stress, all experiments in this chapter were also performed on the THLE-Null cell line which contains the same plasmid less the CYP450 cDNA (section 2.2.1), as a control.

2.3.1 Comparison of cell morphology

The morphology of both cell lines in culture for 24 h was assessed by visual inspection using a microscope at x100 and x200 magnification. Figure 2.5 demonstrated that both THLE-Null and THLE-2E1 were morphologically similar indicating that overexpression of the CYP2E1 enzyme did not have an effect on the physical appearance of the THLE-2E1 cells compared with the THLE-Null.

2.3.2 Comparison of growth rate

The growth rates of both cell lines were also determined by calculating the length of time at which the cell population has doubled in 72 h (section 2.2.4). At 37 °C under 5 % CO_2, conditions under which both cell lines were incubated during culture and treatment, the THLE-Nulls were observed to have a faster growth rate with a doubling time of 29 h compared with a slower growth rate of 42 h observed in the THLE-2E1 cells. This could be indicative of a stress response due to CYP2E1 overexpression having a negative impact on cell growth in the THLE-2E1 cells.

2.3.3 Comparison of mitochondrial activity

Cell viability was chosen as one end-point for assessing the effect of chemical stress in both cell lines using the MTS assay (section 2.2.6). As such, correlations between cell density and mitochondrial activity measured as the change in absorbance at 492 nm (optical density) were also evaluated. As presented in Figure 2.6, both cell lines demonstrated a good correlation between cell and optical densities up to 15,000 cells/well. However, this correlation decreased at cell densities above 15,000 cells/well. Interestingly, this trend was observed in both cell lines suggesting that this effect was not CYP2E1-mediated.

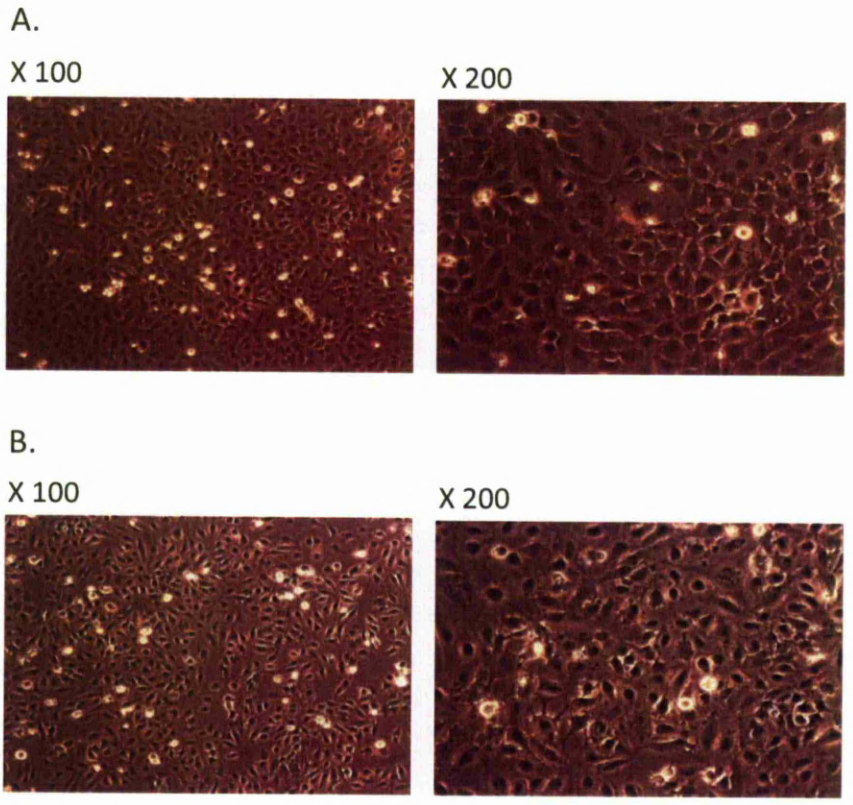


Figure 2.5 Morphological comparison between THLE-Null and THLE-2E1. THLE-Null (A) and THLE-2E1 (B) cell lines were cultured for 24 h in a T25 flask at 250, 000 cells at time 0. Cells were observed under a microscope to determine differences in cell morphology at x100 and x200 magnifications (section 2.2.3).

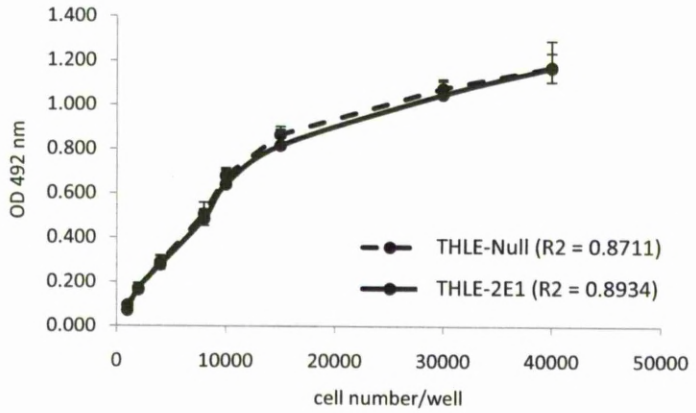


Figure 2.6 Correlation between cell density and mitochondrial function in THLE-Null and THLE-2E1 cells. THLE-Null and THLE-2E1 cells were cultured in a 96 well plate for 24 h at increasing cell densities (0 – 40,000 cell number/well) (section 2.2.5). Mitochondrial activity was determined as a measure of cell viability (section 2.2.6). Correlation was made by plotting the optical density at 492 nm against the cell number per well. Error bars represent the standard error of mean of three independent incubations where each incubation was carried out in triplicate, n = 3.

2.3.4 CYP2E1 protein content in THLE-Null and THLE-2E1 cells

CYP2E1 protein levels were also assessed in both cell lines by western blot as described in section 2.2.11 to demonstrate overexpression of this enzyme by the THLE-2E1 cells. The abundance of CYP2E1 was also compared with cryopreserved human hepatocytes (HL-hep) and human liver homogenate (HL-hom) to determine relative differences in CYP2E1 protein levels between systems.

Figure 2.7-A clearly demonstrates that CYP2E1 was overexpressed in the THLE-2E1 cells compared with the THLE-Null cells. Furthermore, levels of CYP2E1 were similar between the THLE-2E1 cell line and the cryopreserved human hepatocyte samples indicating that both systems were comparable with regards to CYP2E1 protein content. However, levels of CYP2E1 were markedly higher in human liver homogenate compared with the THLE-2E1 cells and cryopreserved human hepatocytes. Interestingly, Ponceau (Figure 2.7-B) and Coomassie (Figure 2.7-C) stains demonstrate marked differences in the protein profile of the THLE cells (CYP2E1 and Null) compared with the cryopreserved human hepatocyte, despite similar levels of CYP2E1. Furthermore, protein profiles were comparable between the cryopreserved human hepatocytes and human liver homogenates despite differences in CYP2E1 protein levels (Figure 2.7-C).

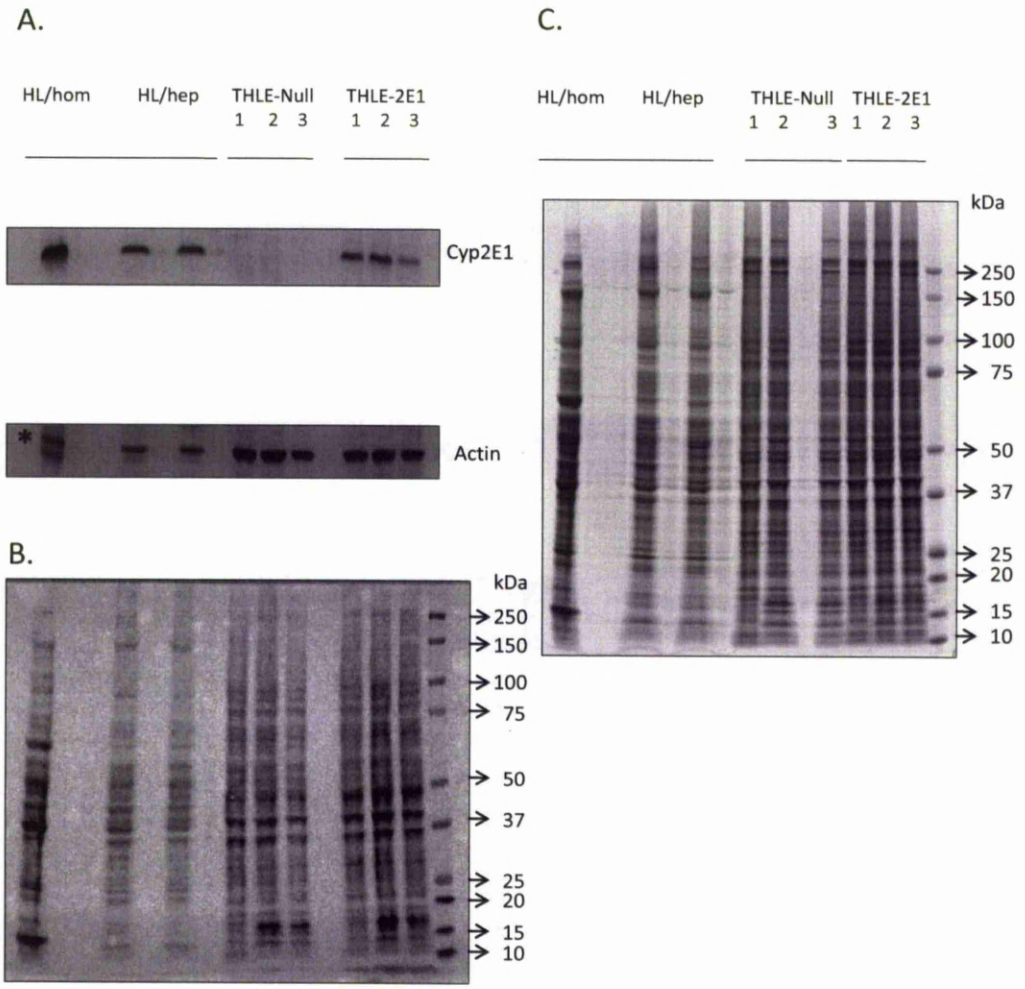


Figure 2.7 CYP2E1 protein content in THLE-Null and THLE-2E1 cell lines. Protein samples (section 2.2.10) were analysed by western blot (A, section 2.2.11) and Coomassie stain (C, section 2.2.12). Cryopreserved human hepatocytes (HL-hep) and human liver homogenate (HL-hom) were used as positive controls for human CYP2E1 protein (56.85 kDa) as well as comparators for differentiating the protein profiles of these three systems. Actin (A) and Ponceau stain (B) were used as controls for sample loading. Numbered lanes (1, 2 and 3) under HL/Hep, THLE-Null and THLE-2E1 represent three separate cultures. Asterisks (*) represent non-specific protein bands.

2.3.5 CYP2E1 activity in the THLE-2E1 cells.

To determine whether the increased CYP2E1 expression in the THLE-2E1 cell line (Figure 2.7) correlated with an increase in activity, both THLE-Null and THLE-2E1 cell lines were incubated with increasing concentrations of chlorzoxazone, a substrate for CYP2E1 metabolism (Figure 2.3) for 2 h (Peter *et al.*, 1990). Detection and quantification of the metabolic product, 6-OH chlorzoxazone was carried out by LC-MS-MS (section 2.2.13). Figure 2.8 shows the detection of 6-OH chlorzoxazone in the THLE-2E1 cells with a

maximum activity of 0.67 nmol/mg/min at 500 µM chlorzoxazone. Higher concentrations of the substrate did not lead to further increase in activity indicating possible saturation of active sites within the CYP2E1 enzyme. Relative to the sensitivity of the assay, no 6-OH chlorzoxazone was detected in the THLE-Null, which is in agreement with the minimal CYP activity reported in these cells (Dambach *et al.*, 2005).

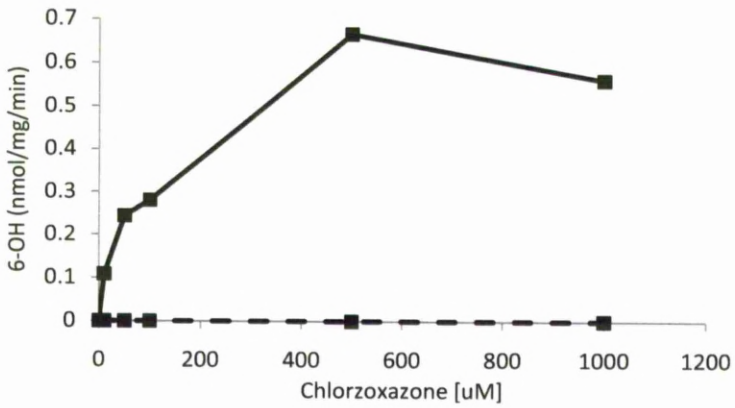


Figure 2.8 THLE-2E1 metabolism of chlorzoxazone to 6-OH chlorzoxazone. THLE-Null (- - - -) and THLE-2E1 (———) cells were incubated with increasing concentrations (0 – 1000 µM) of chlorzoxazone for 2 h. 6-OH chlorzoxazone was detected and quantified by LC-MS-MS (section 2.2.13). Data presented was from one incubation carried out in duplicate (n = 1).

2.3.6 APAP metabolite turnover by THLE-2E1 activity

THLE-2E1 and THLE-Null cells were challenged with APAP, a drug well known to exert its toxicity through CYP2E1 mediated bioactivation to the electrophile NAPQI (Chapter 1 section 1.5.1, Figure 1.2), to assess the potential of THLE-2E1 as an *in vitro* tool for studying xenobiotic-induced chemical stress.

Prior to assessing the toxicological effect of APAP in both cell lines, identification of APAP metabolites was carried out to confirm metabolic turnover. Identification of metabolites was carried out in all samples generated in section 2.2.15 to identify the optimal conditions for APAP bioactivation. The same samples were also analysed for toxicity and GSH levels so that in doing so, correlation between drug bioactivation, drug-toxicity and chemically-induced oxidative stress could be made.

The metabolic profiles of APAP in the THLE-Null and THLE-2E1 cell lines were observed to be distinctly different with the GSH conjugate (P-GSH) detected only in the

THLE-2E1 cells, indicating CYP2E1-mediated bioactivation of APAP into the reactive metabolite NAPQI (Figure 2.9). Furthermore, this also demonstrated a time-dependent increase in CYP2E1-mediated bioactivation of APAP as P-GSH was most abundant at 72 h in the THLE-2E1 cells with the maximum turnover achieved between 5 and 15 mM APAP. A decline in P-GSH conjugate was observed above 15 mM APAP at 72 h suggesting toxicity as a result of prolonged exposure to high concentrations of APAP, an effect not observed at 24 and 48 h.

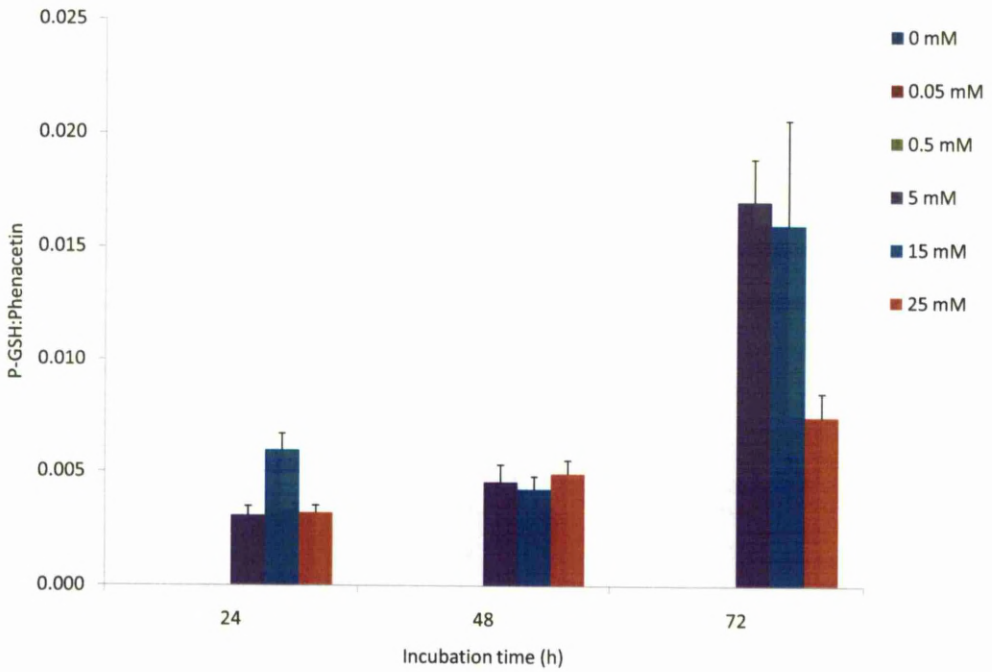
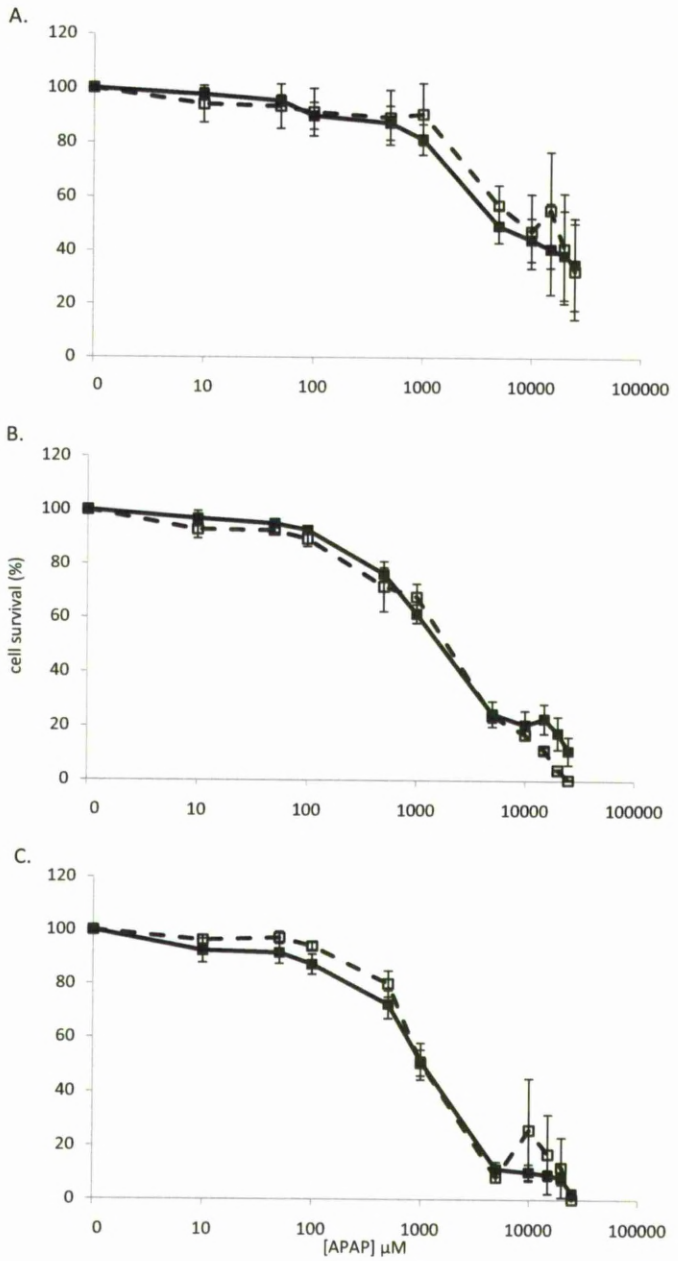


Figure 2.9 THLE-2E1 bioactivation of APAP. THLE-Null and THLE-2E1 cells (15, 000 cells per well, 96 well format) were treated with increasing doses of APAP (0 -25 mM) and incubated for 24, 48 and 72 h, after which samples of the media were analysed by LC-MS-MS (section 2.2.14). Ratios between APAP-GSH conjugate (P-GSH) against the internal standard, phenacetin, were determined. The above data represents the ratio calculated for the THLE-2E1 cells only as no P-GSH conjugate was detected in the THLE-Null. Results presented here were from a single incubation carried out in triplicate (n=1).

2.3.7 APAP-induced toxicity in the THLE-2E1 cells

APAP toxicity was assessed by measuring mitochondrial activity of both THLE-Null and THLE-2E1 cell lines (section 2.2.15). Figure 2.10 represents the percentage survival of both cell lines at increasing concentrations of APAP incubated for 24 (Figure 2.10-A), 48 (Figure 2.10-B) and 72 (Figure 2.10-C) hours. No marked differences in toxicity profiles were

observed between the THLE-Null and THLE-2E1 cell lines, however, the IC_{50} values (Figure 2.10-D) at all time points were lower in the CYP2E1 containing cells which may be suggestive of CYP2E1-mediated APAP toxicity.



D.

	IC_{50} (APAP mM)
Null 24 h	17.89
2E1 24 h	9.74
Null 48 h	2.34
2E1 48 h	1.24
Null 72 h	1.31
2E1 72 h	1.02

Figure 2.10 APAP toxicity in THLE-Null and THLE-2E1 cell lines. THLE-Null (- - - -) and THLE-2E1 (———) cell lines were treated with increasing doses of APAP (0-25 mM) for 24 (A), 48 (B) and 72 (C) h (section 2.2.15). Mitochondrial activity was measured (section 2.2.6) to determine APAP-induced toxicity and represented as percentage of cell survival. IC_{50} values were calculated and summarised in D. Error bars represent standard error of mean of 3 independent experiments in triplicate (n = 3).

2.3.8 Glutathione levels in untreated and APAP-treated cells

GSH content was assessed both before and after exposure to APAP in the THLE-Null and THLE-2E1 cells (section 2.2.9) to determine whether the increase in P-GSH observed in the THLE-2E1 cells at 72 h correlated with changes in GSH levels.

In the absence of APAP, GSH levels appeared higher in the CYP2E1 containing cells at all time points (74.89, 50.62 and 37.91 nmol/mg protein observed at 24, 48 and 72 h respectively) than in the THLE-Null cells (41.98, 35.17 and 29.74 nmol/mg protein observed at 24, 48 and 72 h respectively). The higher level of GSH in the THLE-2E1 cells was maintained at all time points up to a concentration of 15 mM APAP except for that observed at 72 h where GSH was lower in the CYP2E1 containing cells (4.01 nmol/mg protein) compared with the THLE-Null (11.36 nmol/mg protein). However, at 25 mM APAP, GSH was observed to be markedly lower in the THLE-2E1 cells (3.50 nmol/mg protein) than in the THLE-Null cells (31.66 nmol/mg protein) after incubation for 24 h. This marked difference between the cell lines gradually decreased as GSH in the THLE-Null cells decreased at 48 and 72 h (4.79 and 0.65 nmol/mg protein) whilst GSH levels in the THLE-2E1 remained low (0.03 and 1.76 nmol/mg protein observed at 48 and 72 h respectively).

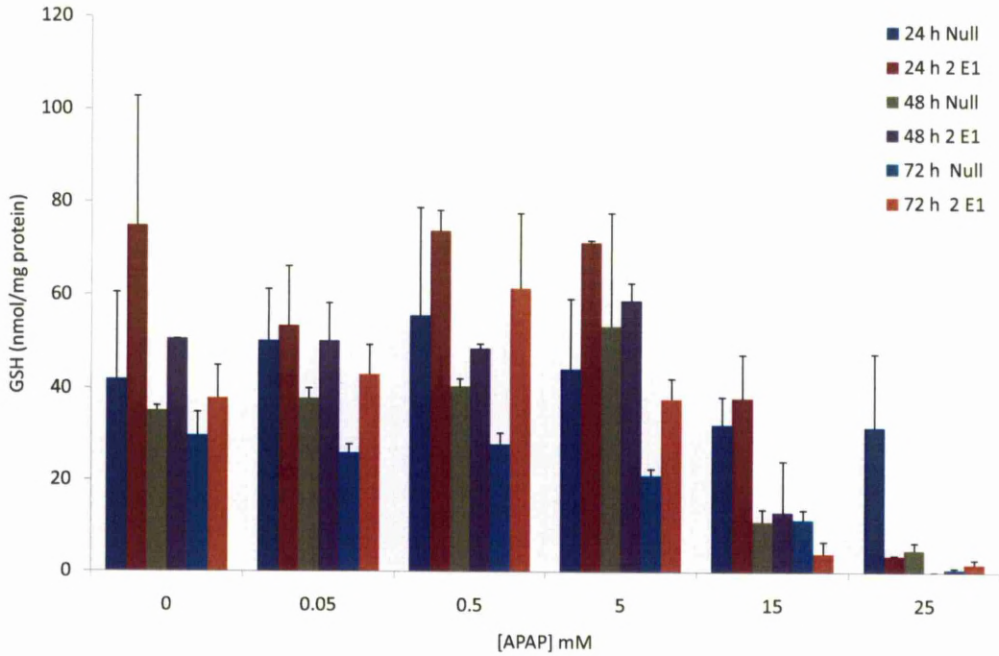


Figure 2.11 GSH levels in THLE-Null and THLE-2E1 cells after a dose and time-dependent treatment with APAP. THLE-Nulls and THLE-2E1 cells were treated with increasing doses (0 – 25 mM) of APAP for 24 (A), 48 (B) and 72 (C) h. Following treatment, GSH and protein contents were measured (sections 2.2.9 and 2.2.8 respectively). Mean GSH data was obtained from a triplicate of two independent experiments (n=2) and represented as nmol/mg protein.

2.4 DISCUSSION

The difficulties in extrapolating toxicity and metabolic profiles of new and existing drugs from pre-clinical studies to the clinic, has led to significant efforts in the development of more accessible alternative test systems such as the THLE-CYP expressing cell lines (Turpeinen *et al.*, 2007). The THLE-2E1 cell line is the main focus of this chapter because of the CYP2E1 enzyme's high capacity to produce ROS in the absence or presence of a metabolic substrate (Gorsky *et al.*, 1984, Ekstrom and Ingelman-Sundberg, 1989), making it an ideal tool for oxidative stress studies. Furthermore, as it is a member of a panel of CYP overexpressing cell lines all of which have originated from the same parental cell line, the THLE-2E1 has an advantage over other CYP2E1 overexpressing cells in having direct comparators against other cell lines expressing other CYP450 isoforms and control. The purpose of the work described in this chapter was therefore to determine whether the THLE-2E1 can provide additional value in the study of oxidative stress. Characterisation of the THLE-2E1 with regards to viability, growth, CYP2E1 expression and metabolic capacity were carried out. This was followed by assessing the THLE-2E1 cell line's potential as a model for studying oxidative stress in the absence or presence of metabolic substrates including chlorzoxazone and the hepatotoxicant APAP, using the THLE-Null as a comparator.

Characterisation of untreated THLE-Null and THLE-2E1 cells in culture demonstrated no differences between the two cell lines with regards to cell morphology (Figure 2.5) and mitochondrial activity (Figure 2.6). However, THLE-2E1 was observed to have a slower growth rate of 42 h compared with the THLE-Null cells of 29 h which may be indicative of a CYP2E1-induced stress response. As mentioned earlier, CYP2E1 is known to have a high capacity for creating an oxidative environment due to its loose coupling with CPR as demonstrated in a variety of test systems (Gorsky *et al.*, 1984, Ekstrom and Ingelman-Sundberg, 1989) including the HepG2-2E1 cell line established in the laboratory of Cederbaum and colleagues, where they have reported levels of ROS to be between 40 – 50 % higher than in the control (Dai *et al.*, 1993, Chen and Cederbaum, 1998, Mari and Cederbaum, 2000). Based on this, the increased protein expression (Figure 2.7) and CYP2E1 activity (Figure 2.8) observed in the THLE-2E1 cells compared with the THLE-Null may have also resulted in higher levels of basal oxidative stress in these cells. In order to adapt to such conditions, cellular defence in the CYP2E1 containing cells may have been enhanced but consequently, bearing a negative impact on the growth of the THLE-2E1 cells. Indeed, this negative effect in cell proliferation was reported in the HepG2-2E1 cells (Chen and Cederbaum, 1998) where the authors associated this observation as a consequence of

CYP2E1 overexpression and reduced levels of vitamin E and ATP. It would therefore be useful if the same measurements (ROS, vitamin E and ATP) were carried out on the THLE-Null and THLE-2E1 cells to determine whether these factors are also the cause of the slow growth rate observed in the THLE-2E1 cells.

CYP2E1 western blot analysis clearly demonstrated overexpression of this protein in the THLE-2E1 cells compared with the THLE-Null (Figure 2.7-A). Furthermore, levels of CYP2E1 were comparable between the THLE-2E1 and cryopreserved human hepatocyte (HL-hep) samples. However, both were lower compared with an equivalent amount of homogenate from a whole human liver (HL-hom). Interestingly, ponceau (Figure 2.7-B) and coomassie (Figure 2.7-C) stains revealed that the overall protein profiles between these three test systems were distinctly different from each other, signifying the importance of caution when utilising such test systems to predict or extrapolate the toxic and metabolic profiles of drugs to a normal human liver. Particularly with the THLE-2E1, being a transformed cell line brings with it the inevitable intrinsic changes required to abrogate the ability to trigger apoptosis and/or necrosis to acquire immortalisation. A feature that makes these cells more amenable for toxic and metabolic studies but it may also be this very feature that prevents direct extrapolation of the toxic and metabolic profiles of drugs to primary hepatocytes. Furthermore, the high oxidative environment as a consequence of CYP2E1 overexpression in the THLE-2E1 cells may have resulted in enhanced cellular defence. This has been demonstrated in the control and APAP-treated THLE-2E1 cells (Figure 2.11) where GSH levels were consistently higher across the three timepoints measured (24, 48 and 72 h) up to and including those incubated with 5 mM APAP compared with the equivalent null cells, a characteristic that would not normally be observed in a normal liver cell. Interestingly, for each APAP concentration used in this particular experiment, a time-dependent decrease in GSH levels was observed for both cell lines. Furthermore, the extent of GSH depletion increases at higher APAP concentration demonstrating an incubation time and APAP concentration effect on GSH levels that is independent to APAP bioactivation.

Despite differences in CYP2E1 expression and overall protein profiles between the THLE-2E1, HL-hep and HL-hom test systems, the distinct CYP2E1 metabolic capacity of the THLE-2E1 cells compared with the THLE-Null as demonstrated in Figure 2.8 with chlorzoxazone gives it potential as a model for investigating the metabolic profile of drugs. No direct comparison of CYP2E1 activity between the THLE-2E1 cells and primary human hepatocytes could be made due to time constraints however, Lahoz *et al.* (2007) have

reported CYP2E1 activity in primary human hepatocytes treated under comparable experimental conditions to that carried out in the THLE-2E1 cells (2 h incubation time, 5 μM chlorzoxazone and similar protein concentration for analysis) and was determined to be 46 pmol/min/mg protein compared with 240 pmol/min/mg protein in the THLE-2E1 cells demonstrating a 5-fold higher metabolic capacity. This to some extent agrees with reports regarding the other CYP-overexpressing THLE cells as they were reported to exhibit higher CYP450 activity compared with primary human hepatocytes (Dambach *et al.*, 2005) but the THLE-2E1 cells certainly do not compare with the magnitude of difference in CYP450 activity (25 – 90 fold observed in other THLE-CYP cells compared with primary human hepatocytes) which could be due to a number of reasons.

Although there is a clear overexpression of the CYP2E1 protein in the THLE-2E1 cells compared with the THLE-Nulls, the level of CYP2E1 overexpression may be lower compared with the level of overexpression of CYP450 enzymes in the other THLE CYP-overexpressing cells. This difference may be a consequence of the high oxidative environment in the THLE-2E1 cells which as suggested earlier may have also affected cell growth, determined to be slower (42 h) compared with the THLE-Nulls (29 h). Cellular resources may have therefore been prioritised for detoxification and/or adaptation to oxidative stress rather than cell growth. CYP2E1 expression may have therefore been compromised. Certainly, this has been demonstrated in the HepG-2E1 cells which were also engineered to overexpress the CYP2E1 enzyme. These cells exhibited significantly higher levels of basal GSH, consistent with observations made in the THLE-2E1 cells (Figure 2.11). Messenger RNA (mRNA) and western blot analysis revealed this elevated GSH in the HepG2-2E1 cells to be associated with the up-regulation of Nrf2 (Mari and Cederbaum, 2000, Nieto *et al.*, 2003), which as mentioned in chapter 1 is an important transcriptional regulator of GCL, the enzyme responsible for the rate-limiting step of GSH synthesis (Kaplowitz *et al.*, 1985, Moinova and Mulcahy, 1999, Wild *et al.*, 1999, Chan and Kwong, 2000, Jeyapaul and Jaiswal, 2000, Reisman *et al.*, 2009c). The role of Nrf2 in this adaptive cell defence mechanism may have also resulted in the up-regulation of phase II and III metabolism as several detoxifying enzymes are also under Nrf2 regulation, including GSTs and MRPs, as detailed in chapter 1. This adaptive mechanism may have also been employed by the THLE-2E1 cells against oxidative stress. Measurement of several endpoints that are under Nrf2 transcriptional regulation in the THLE-2E1 cells compared with the THLE-Nulls would provide a starting point in clarifying the role of Nrf2 in the adaptive defence mechanism acquired by the THLE-2E1 cells against basal and induced cellular stress. Furthermore, the use of another

THLE-CYP overexpressing cell line in this work, such as the THLE-1A2 and/or THLE-3A4, both of which also metabolise APAP could be useful in delineating between CYP2E1 and APAP-induced cellular stress (Dahlin *et al.*, 1984, Raucy *et al.*, 1989, Manyike *et al.*, 2000). Comparisons can also be made in terms of growth, cell viability, CYP450 protein expression, activity and toxicity to determine if the difference seen in the CYP450 activity in the THLE-2E1 cells when compared with primary human hepatocytes (Lahoz *et al.*, 2007) is indeed due to the high oxidative environment. Although the THLE-2E1 cells do not have a comparable CYP450 activity with the other CYP-overexpressing THLE cells, the 5-fold difference in activity compared with that reported in primary human hepatocytes (Lahoz *et al.*, 2007) could still give the THLE-2E1 cell line the potential in predicting the metabolic profile of drugs in humans who exhibit unusual CYP2E1 metabolic activity as a consequence of genetic polymorphism or induction through alcohol exposure/consumption (Prescott, 2000, Schmidt *et al.*, 2002, Krahenbuhl *et al.*, 2007). If indeed the 'lower' difference in CYP2E1 activity of the THLE-2E1 cells compared with the primary human hepatocytes is due to a high oxidative stress environment caused by CYP2E1 overexpression, the THLE-2E1 cells may therefore be comparable with other CYP2E1 overexpressing cell lines such as the HepG2-2E1 cells and could still be a good model for predicting CYP2E1-mediated ADRs *in vitro*.

The CYP2E1-mediated metabolic capacity of the THLE-2E1 cells was further confirmed by the presence of the glutathione adduct (P-GSH) produced as a consequence of APAP bioactivation to the reactive metabolite, NAPQI (Figure 2.9). This gives the THLE-2E1 cell line the potential as a model for investigating not just the metabolic profile of drugs but also their toxic potential. However, when toxicity was measured in these cells upon treatment with varying concentrations of APAP (0-25 mM) and incubation times (24, 48 and 72 h), no significant difference in toxicity profiles was observed between the THLE-2E1 and THLE-Null cells (Figure 2.10). It is plausible that toxicity was not captured under the conditions used. Indeed, although not significant, there was a small but consistent difference in the IC_{50} values between the THLE-2E1 and THLE-Null and this difference was more evident at the 24 h time point. This suggests that although levels of the GSH-P adduct were highest at the 72 h time point, cellular stress/toxicity was more likely to have taken place at 24 h or even earlier. However, due to time constraints, this could not be explored further. The highly similar IC_{50} values between the THLE-Null and THLE-2E1 cells at 48 and 72 h could indicate cellular stress/toxicity from a combination of CYP2E1-mediated bioactivation and accumulation of APAP itself. Of course, this may also just be due to a lack

of activity in the THLE-2E1 cells to cause significant cellular stress and hence toxicity as discussed above.

Work described in this chapter has demonstrated the potential and limitations of the THLE-2E1 cell line as a model for chemical stress. These cells demonstrated comparable characteristics with other CYP2E1 overexpressing liver cell lines such as the HepG2-2E1 cells, particularly in their growth rates, which were determined to be slower compared with their respective controls. Furthermore, the high level of GSH observed was likely to be a consequence of the CYP2E1-mediated oxidative environment. Such feature increases the potential of the THLE-2E1 cell line as a tool for studying oxidative stress. However, these features as a consequence of CYP2E1 overexpression may also be the reason for the limited use of the THLE-2E1 cell lines in predicting the metabolic profile and toxic potential of drugs. Although there is a distinct difference in CYP2E1 activity between the THLE-Null and THLE-2E1 cells, as demonstrated upon incubation with APAP and chlorzoxazone, the level of CYP450 activity is lower compared with other CYP450 expressing THLE cells. This is further supported by the lack of difference in toxicity profiles between the THLE-2E1 and THLE-Nulls. As such, work described in this chapter demonstrated that the THLE-2E1 cell line could be useful in CYP2E1 based metabolism studies but not in elucidating the toxic potential of CYP2E1 substrates.

Chapter 3

The Nrf2$^{(-/-)}$ mouse as an *in vivo* model of chemical stress: effect of acute and chronic exposure to APAP

TABLE OF CONTENTS

3.1 INTRODUCTION

The transcription factor Nrf2 is a well established regulator of the expression of many cytoprotective genes, including NAD(P)H:quinone reductase 1 or NQO1 (Li and Jaiswal 1992a; McMahon *et al.*, 2001a; Ramos-Gomez *et al.*, 2001a; Venugopal *et al.*, 1996; Wang *et al.*, 2006), heme-oxygenase-1 or HO-1 (Alam *et al.*, 1999; Ishii *et al.*, 2000), glutamate cysteine ligase catalytic subunit or GCLC (Wild *et al.*, 1999, Chan and Kwong, 2000, Jeyapaul and Jaiswal, 2000), glutamate cysteine ligase modifier subunit or GCLM (Moinova and Mulcahy, 1999, Wild *et al.*, 1999, Chan and Kwong, 2000), glutathione-S-transferases or GST (Hayes *et al.*, 2000; Knight *et al.*, 2008; Chanas *et al.*, 2002; McMahon *et al.*, 2001) and multidrug resistance-associated proteins or MRPs (Aleksunes *et al.*, 2008b). This regulation is achieved through the binding of Nrf2 to the antioxidant response element (ARE) sequence defined as, 5'GAGTCACAGTGAGTCGGCAAAAATT-3', a cis-acting regulatory element common to the promoter regions of target cytoprotective genes, which in turn induces gene transcription. The importance of this regulatory role has been confirmed by studies carried out in the $Nrf2^{(-/-)}$ mice, whereby various groups have demonstrated that the deletion of the Nrf2 gene leads to a reduction in the constitutive expression and the ability to up-regulate both detoxifying and antioxidant enzymes in response to a chemical insult, resulting in the enhanced susceptibility of these animals to hepatic injury (Chan *et al.*, 2001, Enomoto *et al.*, 2001).

In particular, the two subunits, GCLC and GCLM, which make up glutamate cysteine ligase (GCL), the enzyme responsible for catalysing the rate limiting step of GSH synthesis, are both under the transcriptional regulation of the Nrf2/ARE pathway, as depicted in Figure 3.1. Indeed, it has been well established by our group (Kitteringham *et al.*, 2010) as well as that of others (Klaassen and Reisman, 2010) that the basal level of cellular GSH is between 20-30 % lower in the $Nrf2^{(-/-)}$ mice compared with wild type which is consistent with a reduction in GCL protein activity.

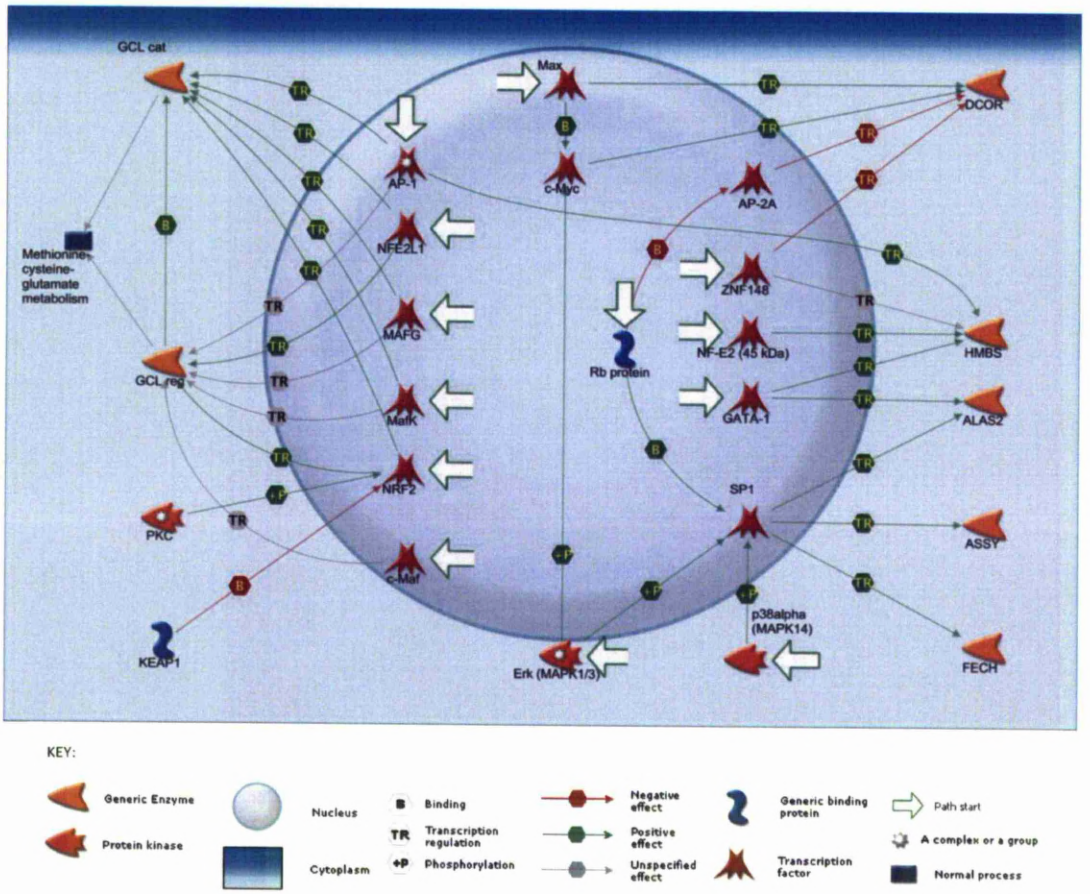


Figure 3.1 The role of Nrf2 in the transcriptional regulation of GSH biosynthesis. Pathways were identified using the MetaCore database describing the role of Nrf2 in amino acid metabolism, including those implicated in GSH synthesis. Abbreviations used in the illustration is summarised in Table 3.1. (MetaCore, GeneGo Inc. http://www.genego.com/metacore.php)

Table 3.1 Abbreviations for Figure 3.1.

ABBREVIATIONS		SPECIES
ALAS2	5-aminolevulinate synthase, erythroid specific, mitochondrial precursor	Human, mouse and rat
AP-1	AP-1	Human, mouse and rat
AP-2A	Transcription factor AP-2A alpha	Human, mouse and rat
ASSY	Argininosiccinate synthase	Human, mouse and rat
c-Maf	Transcription factor Maf	Human, mouse and rat
DCOR	Ornithine decarboxylase	Human, mouse and rat
Erk(MAPK1/3)	Erk (MAPK1/3)	Human, mouse and rat
FECH	Ferrochelatase	Humand and mouse
	Mitochondrial precursor ferrochelatase (predicted)	Rat
GATA-1	Erythroid transcription factor	Human, mouse and rat
GCL cat	Glutamamte-cysteine ligase catalytic subunit	Human, mouse and rat
GCL reg	Glutamate-cysteine ligase regulatory subunit	Human, mouse and rat
HMBS	Porphobilinogen deaminase	Human, mouse and rat
KEAP1	Kelch-like ECH-associated protein 1	Human, mouse and rat
MafK	Transcription factor MafK	Human and mouse
	Transcription factor v-maf musculoaponeurotic fibrosarcoma oncogene family, protein K (avian)	Rat
MAFG	Transcription factor MAFG	Human and mouse
	Transcription facto v-maf musculoaponeurotic fibrosarcoma oncogene family, protein G (avian)	Rat
Max	Protein Max	Human, mouse and rat
NF-E2 (45 kDa)	Transcription factor NF-E2 45 kDa subunit	Human, mouse and rat
NFE2L1	Transcription factor nuclear factor erythroid 2-related factor 1	Human and mouse
	Transcription factor nuclear factor, erythroid derived 2 –like 1 (predicted)	Rat
Nrf2	Transcription factor nuclear factor erythroid 2-related factor 2	Human, mouse and rat
P38alpha (MAPK14)	Mitogen-activated protein kinase 14	Human, mouse and rat
PKC	PKC	Human, mouse and rat
SP1	Transcriptional factor SP1	Human, mouse and rat
ZNF148	Zinc finger protein 148	Human, mouse and rat

GSH plays a key role in maintaining the redox status of the cell as well as providing cellular defence against chemical stress through the detoxification of both electrophiles and ROS. In addition to the transcriptional regulation of GCL by Nrf2, several other important factors also influence the synthesis of GSH. One such factor is the negative feedback loop that is governed by the cellular concentration of GSH. This mechanism influences the up- or down-regulation of GCL activity and hence GSH synthesis (Richman and Meister, 1975). Another factor is the availability of precursors, in particular, of the thiol containing amino acid cysteine. The thiol group of this molecule is key in the detoxification mechanism employed by GSH therefore cysteine availability is crucial for both the synthesis and function of GSH (Kaplowitz et al., 1985; Richman and Meister 1975; Meister and Anderson 1983). Heterodimerisation of GCLC and GCLM is another factor that could influence GSH synthesis (Seelig *et al.*, 1984, Huang *et al.*, 1993a, Huang *et al.*, 1993b).

As demonstrated in Figure 3.2, the supply of cysteine for GSH synthesis is dependent on a number of pathways involved in amino acid metabolism. These include the methionine, transsulfuration, polyamine and cysteine sulfinate pathways (Finkelstein 1998; Lu 1999; Tarver and Schmidt 1939). As cysteine is used by GCL as substrate for the production of γ-glutamylcysteine (γ-GC), these pathways are also implicated in the transcriptional regulation of GCL by Nrf2. Furthermore, polyamine metabolism has been reported to induce directly the transcription of phase II detoxifying enzymes through the activity of H_2O_2 and aminoaldehydes which are products of spermidine oxidation, on the Nrf2/ARE pathway (Kwak *et al.*, 2003).

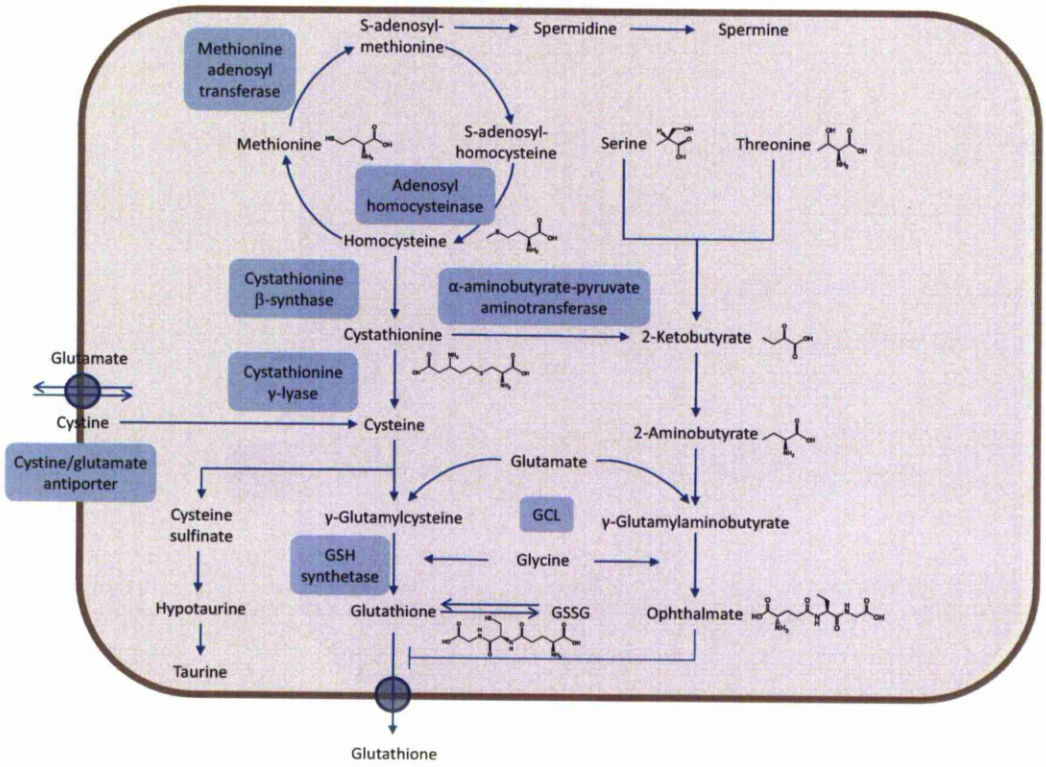


Figure 3.2 The glutathione metabolome. A schematic diagram illustrating the GSH biosynthetic pathway including peripheral pathways implicated in this process. For a more detailed version, refer to chapter 1, Figure 1.6 and Appendices 1 – 4.

As mentioned earlier, in addition to the regulatory role of Nrf2 in the constitutive expression of various detoxifying and antioxidant genes, it is also responsible for the adaptive response of these genes against chemical stress. Due to its nucleophilic nature, high levels and cellular disposition, GSH is at the forefront of the detoxification of both electrophiles and ROS produced during chemical stress. However as GCL is regulated by Nrf2, this cytoprotective role is highly dependent on the response of Nrf2 to chemical stress. Amongst many hepatotoxicants, APAP is a well known modulator of GSH. It is widely available as an over-the-counter antipyretic analgesic that is safe for use at normal therapeutic doses. However, it is also the leading cause of drug-induced liver injury in the UK (Davern *et al.*, 2006) and United States (Larson et al., 2005; Lee 2004). To date, it is widely accepted that the molecular mechanism of APAP hepatotoxicity is initiated by the cytochrome P450-mediated bioactivation of APAP to the reactive metabolite NAPQI (Mitchell *et al.*, 1973b, Dahlin *et al.*, 1984, Lee *et al.*, 1996).

At therapeutic doses, APAP primarily undergoes glucuronidation and sulfation metabolism prior to its excretion in the urine. NAPQI is also formed in small quantities (5 – 10 % APAP) mainly by CYP2E1 bioactivation and to a lesser extent by CYP1A2 and CYP3A4 (Dahlin *et al.*, 1984, Raucy *et al.*, 1989, Manyike *et al.*, 2000) but is spontaneously quenched through GSH conjugation and subsequently excreted in the urine as mercapturic acid or cysteine conjugate (Mitchell *et al.*, 1973b). However, these detoxification pathways can be overwhelmed as a consequence of APAP overdose, resulting in large amounts of NAPQI being produced which cannot be efficiently detoxified as stated above. As a result, the cellular store of GSH becomes depleted enabling NAPQI to covalently bind to macromolecules and/or to generate ROS which consequently leads to hepatotoxicity (Jollow *et al.*, 1973, Potter *et al.*, 1973).

The hepatotoxic effect of APAP is commonly associated with the administration of an acute toxic dose as observed in the clinic (Lee, 2004, Larson *et al.*, 2005), and demonstrated in model systems (Kitteringham *et al.*, 2000, Randle *et al.*, 2008). However, there is evidence that prolonged exposure to APAP may lead to reduced sensitivity to hepatotoxicity. In a case study described by Shayiq *et al.*, (1999), a physician who was addicted to oxycodone presented no sign of liver injury despite ingesting up to 36,000 tablets for 2 years, equivalent to a daily dose of 65 g APAP. Similarly, a patient suffering from rheumatoid arthritis who took daily doses of 15 -20 g APAP as pain relief for 5 years showed no signs of APAP toxicity. In this particular study, the authors attributed this resistance to a combination of slow APAP absorption, enhanced detoxification and reduced drug bioactivation compared with normal volunteers (Tredger *et al.*, 1995).

Following from this, *in vivo* animal studies have been undertaken to simulate the cases described above and have reported similar findings. Shayiq *et al.* (1999) demonstrated that exposure of mice to incremental doses of APAP afforded these animals protection against a subsequent lethal dose. The protection was attributed to increased cell proliferation within the liver and reduced covalent binding as a result of a shift in the bioactivation of APAP from centrilobular to periportal regions, where CYP2E1 is not found and GSH is more abundant. In a further study by Aleksunes *et al.* (2008), the protective mechanism was reported to involve the induction of a member of the multidrug resistance-associated protein family, MRP4.

Whilst the precise mechanism underlying the adaptive change resulting in protection against hepatotoxicity following prolonged APAP exposure remains undefined,

the possible explanations proposed point to a generalised up-regulation of cellular defence and a reduced capacity to bioactivate the drug. Furthermore, the enhanced cellular proliferation observed in the Shayiq study (1999) suggests that the liver is able to repair tissue damage more readily in the treated mice. Studies conducted within the department and elsewhere have identified Nrf2 as a regulator of both cellular defence and of cell viability. Several of the key mechanisms identified in the chronic studies described above, such as GSH synthesis, cytochrome P450 activity, cellular transport and cell proliferation are modulated by the Nrf2/ARE pathway. It is thus plausible that Nrf2 may be a principal regulator of the adaptive response induced by prolonged exposure to APAP. Consequently, the studies described in this chapter were designed to test the hypothesis that Nrf2 is essential in the adaptive response against acute and chronic exposure to chemical stress inducers. This hypothesis was tested by comparing the ability of $Nrf2^{(+/+)}$ and $Nrf2^{(-/-)}$ mice to adapt to APAP treatment following the dosing regimens summarised in Table 3.2. Furthermore, we investigated the underlying basis of this protection through a targeted metabolomic analysis focussing on the GSH system by selecting and monitoring perturbations of key metabolites in this system. Substantiation of this hypothesis raises the possibility that mice deficient in the Nrf2-driven cellular defence system could provide a valuable *in vivo* model of oxidative and drug-induced chemical stress.

3.2 MATERIALS AND METHODS

3.2.1 Materials

All chemicals and reagents were purchased from Sigma Aldrich Chemical Co. (Poole, Dorset, UK) unless otherwise stated. Acetone and formic acid were purchased from VWR (Lutterworth, Leicestershire, UK). Sodium hydroxide and methanol were purchased from Fisher Scientific (Loughborough, Leicestershire, UK). Ophthalmic acid was purchased from Bachem (Weil am Rhein, Germany). Infinity ALT (GPT) liquid stable reagent was purchased from Thermo Scientific (Loughborough, Leicestershire, UK).

3.2.2 Animal dosing regimen

All animal experiments were undertaken in accordance with criteria outlined in a license granted under the Animals (Scientific Procedures) Act 1986, and approved by the Animal Ethics Committees of the University of Liverpool. The generation of the $Nrf2^{(-/-)}$ mice have been previously described (Itoh *et al.*, 1997, McMahon *et al.*, 2001). All animals were housed under 12-h light/dark cycles with room temperature maintained between 19 °C – 23 °C. Animals had free access to food and water.

3.2.3 The effect of acute APAP treatment to the GSH system in wild type and $Nrf2^{(-/-)}$ mice

Male C57BL/6 $Nrf2^{(+/+)}$ and $Nrf2^{(-/-)}$ mice were administered intraperitoneally (i.p.) with a sub-lethal dose of 350 mg/kg APAP in 0.9 % saline or vehicle (control). Animals were sacrificed at 0, 1, 3 and 5 h (Table 3.2) by carbon dioxide (CO_2) asphyxiation and cervical dislocation. Livers were removed and snap frozen immediately in liquid nitrogen and stored at -80 °C until further use. Serum was obtained by allowing the blood to clot at room temperature for 1 h followed by pulse centrifugation at 4000 rpm (Bio-Tek FL600 Vermont, USA).

3.2.4 The effect of chronic APAP treatment at escalating doses to the GSH system in wild type and Nrf2$^{(-/-)}$ mice

Male C57BL/6 mice were administered with escalating doses of APAP (150 – 600 mg/kg) in 0.9 % saline or vehicle (control; i.p.) for 8 consecutive days as shown in Table 3.2. Animals were killed 5 h after the last dose at day 8 by CO_2 asphyxiation and cervical dislocation. Sample collection and storage were as detailed in section 3.2.3.

3.2.5 The effect of chronic APAP treatment at escalating doses followed by a lethal dose to the GSH system in wild type and Nrf2$^{(-/-)}$ mice

Male C57BL/6 mice were administered escalating doses of APAP (150, 150, 300, 300, 450, 450, 600 and 600 mg/kg) in 0.9 % saline or vehicle (control i.p.) for 8 consecutive days as shown in Table 3.2, followed by a 1000 mg/kg APAP dose in 0.9 % saline or vehicle (control) on day 9 for 5 h. Animals were killed by CO_2 asphyxiation and cervical dislocation. Sample collection and storage were as detailed in section 3.2.3.

Table 3.2. Summary of studies carried out in this chapter which were designed to test the hypothesis that Nrf2 is essential in the adaptive response against chemical stress. ***Unchallenged*** refers to chronic treatment with APAP up to day 8, without the challenge dose of 1000 mg/kg APAP on day 9, whereas ***challenged*** refers to chronic treatment including day 9.

TYPE OF STUDY	**DOSING REGIMEN**								
Acute	350 mg/kg APAP treatment for 0, 1, 3 and 5 h.								
Chronic	Single ip dose/day (mg/kg)								
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9
Unchallenged	150	150	300	300	450	450	600	600	
Challenged	150	150	300	300	450	450	600	600	1000

3.2.6 Determination of serum alanine transaminase (ALT) levels

Blood from the above animals (sections 3.2.3 – 3.2.5) were allowed to clot overnight at 4 °C and subsequently pulse centrifuged at 4000 rpm (Bio-Tek FL600 Vermont, USA) after which serum samples were collected. Determination of serum ALT was carried out using Infinity ALT (GPT) reagent (Thermo Scientific, Loughborough, UK) according to the manufacturer's instructions.

3.2.7 Determination of hepatic GSH content

Hepatic GSH was determined based on the method of (Vandeputte *et al.*, 1994). Briefly, approximately 50 mg of tissue was homogenised in 5-sulfosalicyclic acid (SSA; 200 µl; 6.5 % w/v) and GSH stock buffer (800 µl; 143 mM NaH_2PO_4, 6.3 mM EDTA), pH 7.4). Protein was precipitated on ice for 10 min prior to centrifugation (14000 rpm for 5 min). Total hepatic GSH content was measured (412 nm) according to the kinetic reaction between GSH and GSH reductase on a microtitre-plate reader (UV-visible absorbance MRX Dynatech Laboratories, Chantilly, VA).

3.2.8 Determination of protein concentration

Hepatic protein content was determined according to the method of Lowry (1951) as described in chapter 2, section 2.2.8.

3.2.9 Metabolite analysis by LC-MS-MS

3.2.9.1 Homogenisation of liver tissue

Liver tissue from each animal was weighed and homogenised in phosphate buffer (1:5; w/v) using a micropestle, followed by a freeze (– 80 °C for 1 h)/ thaw (room temperature) step and sonication (Soniprep 150, MSE London, UK, three times at ten second bursts) before centrifugation for 30 min at 4 °C. The supernatants were collected and stored at -80 °C until required.

3.2.9.2 Preparation of metabolite standard stocks

Combined metabolite standards with a concentration range of 0 – 4 µM were prepared in 0.1 % formic acid. A 1 mL solution of 10 mM internal standard (*S*-hexylglutathione) was prepared by firstly adding 450 µl 0.1 % formic acid and 50 µl 1 M NaOH to allow the *S*-hexylglutathione to dissolve to the correct pH. An additional 500 µl 0.1 % formic acid was added to give a concentration of 10 mM and was then further diluted to obtain a 5 µM stock.

3.2.9.3 Preparation of standard and sample mixtures for analysis.

A standard mixture was prepared by adding 10 µl metabolite standard, 10 µl 5 µM *S*-hexylglutathione stock solution and 10 µl 30 mg/ml BSA (in dH_2O) and made up to 50 µl with dH_2O. The sample mixture consisted of 10 µl liver homogenate or serum with 10 µl 5 µM *S*-hexylglutathione and made up to 50 µl with dH_2O.

For the determination of cysteine, a derivatisation step was carried out with iodoacetamide to stabilise the sulfhydryl group within the cysteine molecule. Derivatised standard mixture consisted of 5 µl standard, 10 µl 5 µM *S*-hexylglutathione, 5 µl 30 mg/ml BSA (in dH_2O) and 30 µl 550 mM iodoacetamide (dissolved in 10 mM ammonium bicarbonate) and allowed to react at room temperature for 30 min. The derivatised sample mixture consisted of 5 µl liver homogenate or serum, 10 µl 5 µM *S*-hexylglutathione, 5 µl dH_2O and 30 µl 550 mM iodoacetamide. Samples and standards were de-proteinated with 200 µl ice-cold acetone at -20 °C for 1 h. Supernatants were obtained by centrifugation (Beckman GS-15R High Wycombe, Buckinghamshire, UK) for 10 mins at 4 °C. Acetone was removed by drying the supernatants down to approximately 50 µl in a Speed Vac (Eppendorf Concentrator 5301 Histon, Cambridge, UK) at 30 °C for 25 min. Samples were subsequently diluted with 200 µl 0.1 % formic acid and stored at 4 °C prior to analysis.

3.2.9.4 LC-MS-MS

All LC-MS-MS experiments were performed using a Perkin Elmer SCIEX API 2000 LC-MS-MS system with a Perkin Elmer Series 200 Autosampler and HPLC Pump (Massachusetts, USA). Analytes were separated using a Prodigy C18 column 5 µ ODS (2), 150 x 4.6 mm length (Phenomenex Macclesfield, Cheshire, UK).

The flow-rate of 1 mL/min was achieved with an elution gradient composed of solvent A (0.1 % formic acid in dH_2O) and solvent B (100 % MeOH). The gradient started with a 12 min linear gradient from 100 % – 40 % solvent A, followed by an isocratic step of 40 % solvent A for 2 min, a linear gradient from 40 % to 100 % solvent A for 8 min and then a final isocratic step of 100 % solvent A for 8 mins. The total analysis time and injection volume were 35 min and 50 µl respectively. The autosampler syringe was subjected to three times pre and post sample injection washes with solvent A. Analytes were detected in the positive ion mode using MS-MS with MRM which uses the unique fragmentation pattern of each metabolite for detection. Optimisation of the fragmentation pattern and quantification of metabolites were carried out using reference standards. Optimised MRM fragmentation patterns used to detect the metabolites are summarised in Table 3.3. Nebuliser, curtain and collision gases were set to 20, 10 and 8 respectively. Ionisation spray was set at 5.5 kV and the source temperature was 300 °C. System control and data acquisition were performed using Analyst Version 1.4 software.

Table 3.3 Optimised MRM transitions used for the detection and quantification of each analyte.

Compound	Parent Ion (m/z)	Fragment Ion (m/z)
Ophthalmic acid	290	58
GSSG	613	355.3
Methionine	149.66	104.3
Spermidine	146.109	71.9
Taurine	125.94	108.3
Cysteine	179.66	88.2
S-hexylglutathione	392.076	246.5

3.2.10 Statistical analysis

All results are expressed as mean ± standard error of the mean (SEM). All values were analyzed for non-normality using the Shapiro-Wilk test. A student's unpaired t test or ANOVA was used if normality was indicated. For non-normally distributed data a Mann Whitney U or a Kruskal Wallis tests was applied. Results were considered significant when p values were less than 0.05.

3.3 RESULTS

3.3.1 Metabolic perturbations within the GSH system in response to an acute sub-lethal dose of APAP in $Nrf2^{(+/+)}$ and $Nrf2^{(-/-)}$ mice

To determine the role of Nrf2 in the adaptive response to APAP-induced stress, $Nrf2^{(+/+)}$ and $Nrf2^{(-/-)}$ mice were treated with an acute dose of 350 mg/kg APAP for 0, 1, 3 and 5 h. Liver and serum samples were collected and analysed for serum ALT levels as a measure of liver damage as well as metabolites within the GSH system to deteremine the relationship between Nrf2 and perturbations within the GSH system in response to APAP-induced stress. Serum ALT levels were also measured as detailed in section 3.2.6 to indicate liver injury and/or toxicity as a consequence of acute paracetamol treatment as described above.

3.3.1.1 Determination of hepatic injury by measurement of serum ALT

Serum ALT was measured to determine whether an acute dose of 350 mg/kg APAP leads to liver injury and/or toxicity in the $Nrf2^{(-/-)}$ mice compared with $Nrf2^{(+/+)}$ after a 5 h period post dose.

At 3 h post dose, serum ALT was detected to be significantly higher in both $Nrf2^{(+/+)}$ and $Nrf2^{(-/-)}$ mice (318.31 ± 199.17 and 215.15 ± 92.58 U/L respectively) compared with their corresponding control groups (25.46 ± 6.31 and 41.60 ± 19.46 U/L for $Nrf2^{(+/+)}$ and $Nrf2^{(-/-)}$ respectively) and the 0 h control animals (15.19 ± 2.77 and 13.95 ± 2.34 U/L for $Nrf2^{(+/+)}$ and $Nrf2^{(-/-)}$ respectively) (Figure 3.3-A and B respectively). After 5 h, the difference in ALT values was markedly higher in the treated $Nrf2^{(-/-)}$ animals (2019.93 ± 1315.93 U/L) compared with the time-matched control group (30.30 ± 14.48 U/L) and was determined to be significantly higher compared with ALT levels measured in the 0 h control group (13.95 ± 2.34 U/L). On the other hand, ALT levels in the equivalent wild type group (234.47 ± 141.66 U/L) were only marginally higher above baseline values (30.39 ± 14.49 U/L for time-matched control), but demonstrated significantly higher ALT when compared with the 0 h Nrf2(+/+) control group (15.19 ± 2.77 U/L). These data indicate a potential adaptive response induced by the $Nrf2^{(+/+)}$ mice to the acute APAP treatment of 350 mg/kg allowing the attenuation of further liver injury at 5 h. On the other hand, the ability of the $Nrf2^{(-/-)}$

mice to launch an adaptive response may have been abrogated resulting in higher serum ALT at 5 h compared with time-matched control.

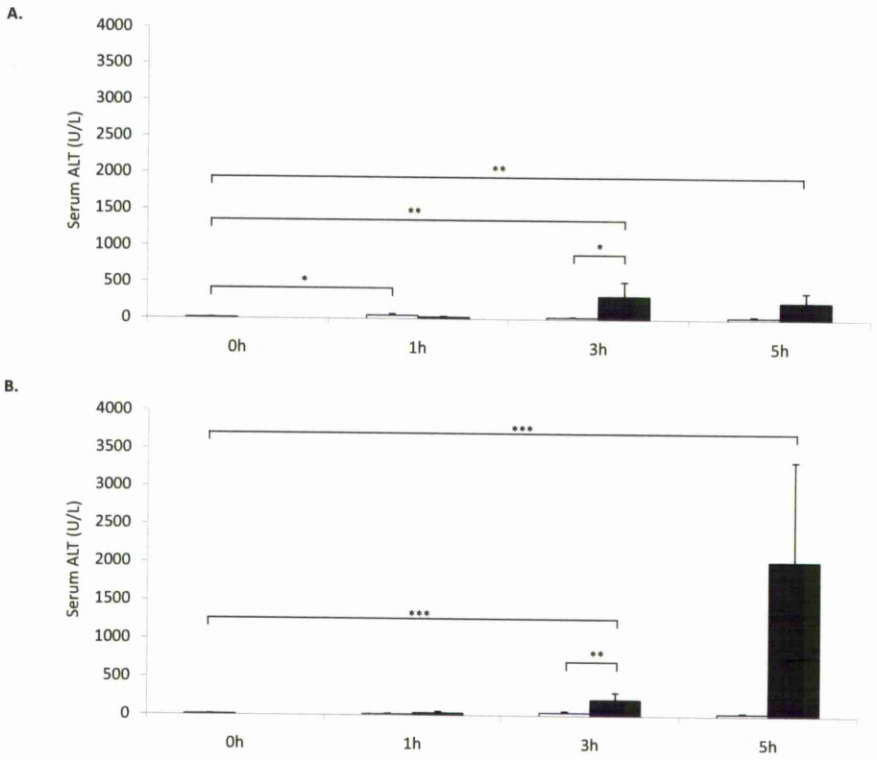


Figure 3.3 Serum ALT activities in mice after treatment with an acute dose of APAP. Male C57BL/6 $Nrf2^{(+/+)}$ (A) and $Nrf2^{(-/-)}$ (B) mice were administered an acute dose of 350 mg/kg APAP (■) or vehicle (□). Blood samples were collected at 0, 1, 3 and 5 h and serum extracted as detailed in section 3.2.3. Serum ALT activity was measured using the protocol detailed in section 3.2.6. Data from both A and B are presented as mean serum ALT activity (U/L) ± SEM (n = 4-6 animals).

3.3.1.2 Metabolite analysis by LC-MS-MS

3.3.1.2.1 Metabolite profile of $Nrf2^{(+/+)}$ and $Nrf2^{(-/-)}$ mice in response to an acute dose of APAP

Figures 3.4 (hepatic) and 3.5 (serum) represent the effect of acute APAP treatment on the GSH system at the four time-points measured (0, 1, 3 and 5 h) in the $Nrf2^{(+/+)}$ and $Nrf2^{(-/-)}$mice.

Acute treatment in the $Nrf2^{(+/+)}$ mice with 350 mg/kg APAP resulted in a significantly lower level of hepatic GSH at 1 h post dose (7.51 ± 2.27 nmol/mg protein) compared with time-matched control (32.83 ± 7.48 nmol/mg protein). Although not determined to be significant, this trend was consistent at 3 and 5 h with hepatic GSH also lower in the $Nrf2^{(+/+)}$ APAP-treated groups (17.37 ± 4.56 and 36.88 ± 5.70 nmol/mg protein respectively) compared with their time-matched controls (28.04 ± 3.90 and 56.63 ± 13.07 nmol/mg protein respectively). However, hepatic GSH was observed to be generally higher in the $Nrf2^{(+/+)}$ APAP-treated animals at the later time-points of 3 and 5 h compared with those from the 1 h group potentially indicating GSH recovery.

Similar to observations made in the $Nrf2^{(+/+)}$mice, hepatic GSH in the APAP-treated $Nrf2^{(-/-)}$ animals were significantly lower at 1, 3 and 5 h (9.28 ± 3.15, 4.94 ± 1.16 and 14.62 ± 6.93 nmol/mg protein respectively) compared with time-matched control groups (38.13 ± 7.31, 28.36 ± 2.42 and 33.69 ± 5.39 nmol/mg protein respectively). When comparing the level of hepatic GSH between the APAP-treated $Nrf2^{(+/+)}$ and $Nrf2^{(-/-)}$ at 3 and 5 h, GSH levels were generally higher in the $Nrf2^{(+/+)}$ animals (17.36 ± 4.56 and 36.88 ± 5.70 nmol/mg protein at 3 and 5 h respectively) compared with the $Nrf2^{(-/-)}$ mice (4.94 ± 1.16 and 14.62 ± 6.93 nmol/mg protein at 3 and 5 h respectively). This difference could be indicative of the difference in which these two strains were able to up-regulate GSH synthesis. Furthermore, hepatic GSH in the $Nrf2^{(+/+)}$ control group at 0 h (30.05 ± 5.95 nmol/mg protein) was only significantly higher when compared with levels measured in the $Nrf2^{(+/+)}$ APAP-treated group at 1 h, whilst all $Nrf2^{(-/-)}$ APAP-treated groups at 1, 3 and 5 h were significantly lower compared with the $Nrf2^{(-/-)}$ control group at 0 h (42.65 ± 6.30 nmol/mg protein).

Similar to the pattern observed with hepatic GSH, GSSG in the liver was also lower at 1, 3 and 5 h in the $Nrf2^{(+/+)}$ APAP-treated groups (1.62 ± 0.40, 7.27 ± 2.25 and 11.79 ±

1.82 nmol/mg protein respectively) compared with time-matched controls (15.03 ± 2.10, 19.34 ± 3.80 and 23.09 ± 4.04 nmol/mg protein respectively). Furthermore, levels of hepatic GSSG in these animals were also significantly lower compared with the 0 h $Nrf2^{(+/+)}$ control group (23.06 ± 2.62 nmol/mg protein). However, a trend towards increasing levels of GSSG was observed in the $Nrf2^{(+/+)}$ APAP-treated groups over the 1, 3 and 5 h time points suggesting that the level of chemical stress may have started to subside at the later time points (3 and 5 h) allowing GSH levels to recover and become available for physiological oxidation reactions.

Hepatic GSSG was also markedly lower at 1, 3 and 5 h in the $Nrf2^{(-/-)}$ APAP-treated groups (3.32 ± 1.10, 1.91 ± 0.99 and 5.47 ± 1.72 nmol/mg protein respectively) compared with the 0 h control group (12.57 ± 2.53 nmol/mg protein) as well as their corresponding time-matched control groups, particularly at 1 and 3 h (10.55 ± 1.52 and 13.22 ± 1.58 nmol/mg protein respectively). Hepatic GSSG was significantly lower in the $Nrf2^{(-/-)}$ control group at 0 and 5 h (12.57 ± 2.53 and 9.18 ± 2.35 nmol/mg protein) compared with their $Nrf2^{(+/+)}$counterparts (23.06 ± 2.62 and 23.09 ± 4.04 nmol/mg protein). This was also observed in the APAP-treated groups where the $Nrf2^{(-/-)}$ animals at 5 h demonstrated significantly lower levels of hepatic GSSG (5.47 ± 1.72 nmol/mg protein) compared with this group's wild type counterpart (11.79 ± 1.82 nmol/mg protein).

GSSG in the serum of the $Nrf2^{(+/+)}$ APAP-treated animals at 1 and 3 h were significantly lower (1.89 ± 0.88 and 13.97 ± 5.83 μM respectively) compared with the 0 h wild type control (23.80 ± 8.68 μM). Furthermore, GSSG in the serum of the 1 h $Nrf2^{(+/+)}$ APAP-treated animals were significantly lower (1.89 ± 0.88) compared with the corresponding time-matched control group (9.80 ± 3.21 μM). Only serum GSSG measured in the $Nrf2^{(-/-)}$ APAP-treated animals at 1 h (2.38 ± 2.75 μM) demonstrated a significant difference where it was lower compared with the 0 h $Nrf2^{(-/-)}$ control group (13.86 ± 5.25 μM).

A time-dependent increase was observed in hepatic ophthalmic acid measured in the $Nrf2^{(+/+)}$ APAP-treated animals where average levels at 1, 3 and 5 h were significantly higher (0.30 ± 0.04, 0.37 ± 0.07 and 0.68 ± 0.20 nmol/mg protein respectively) compared with their time-matched controls (0.14 ± 0.01, 0.2 ± 0.08 and 0.21 ± 0.08 nmol/mg protein respectively). Furthermore, hepatic ophthalmic acid in the $Nrf2^{(+/+)}$ APAP-treated animals at 1, 3 and 5 h were also significantly higher when compared with the $Nrf2^{(+/+)}$ 0 h control group (0.16 ± 0.01 nmol/mg protein). On the other hand, although hepatic ophthalmic acid

in the APAP-treated $Nrf2^{(-/-)}$ were consistently higher compared with their time-matched controls, these differences were not determined to be significant. Interestingly, hepatic ophthalmic acid in the $Nrf2^{(-/-)}$ mice at 1 h (0.16 ± 0.03 nmol/mg/protein) was significantly lower compared with its wild type equivalent (2.12 ± 0.61 nmol/mg/protein).

Ophthalmic acid levels in the serum of APAP-treated $Nrf2^{(+/+)}$ mice were significantly higher at 3 and 5 h (6.24 ± 1.12 and 9.01 ± 2.4 μM respectively) compared with their time-matched controls (1.01 ± 0.17 and 0.44 ± 0.25 μM respectively) and the 0 h $Nrf2^{(+/+)}$ control group (2.08 ± 0.41 μM). Only at 3 h was there a significant difference observed in the levels of serum ophthalmic acid in the $Nrf2^{(-/-)}$ animals between the APAP-treated (3.98 ± 1.16 μM) and time-matched control (1.19 ± 0.36 μM) groups.

Significant differences in hepatic cysteine were only observed at the 3 h timepoint. APAP-treated $Nrf2^{(+/+)}$ (0.11 ± 0.03 nmol/mg protein) and the 3 h $Nrf2^{(-/-)}$ control animals (0.08 ± 0.01 nmol/mg protein) demonstrated significantly lower hepatic cysteine levels compared with the 3 h wild type control group (0.26 ± 0.05 nmol/mg protein).

Significant differences were also observed in serum methionine levels. Serum methionine in the $Nrf2^{(-/-)}$ control group at 0 h (9.01 ± 0.86 μM) was significantly lower compared with its wild type counterpart (37.20 ± 8.00 μM) and the $Nrf2^{(-/-)}$ control groups at 1, 3 and 5 h (20.33 ± 8.68, 19.35 ± 5.77 and 33.47 ± 12.29 μM respectively). No significant differences were observed in hepatic spermidine and taurine levels as well as serum spermidine. However, serum taurine in the $Nrf2^{(-/-)}$ animals at 0 h was significantly lower (199.14 ± 20.45 μM) compared with its wild type counterpart (271.90 ± 8.10 μM).

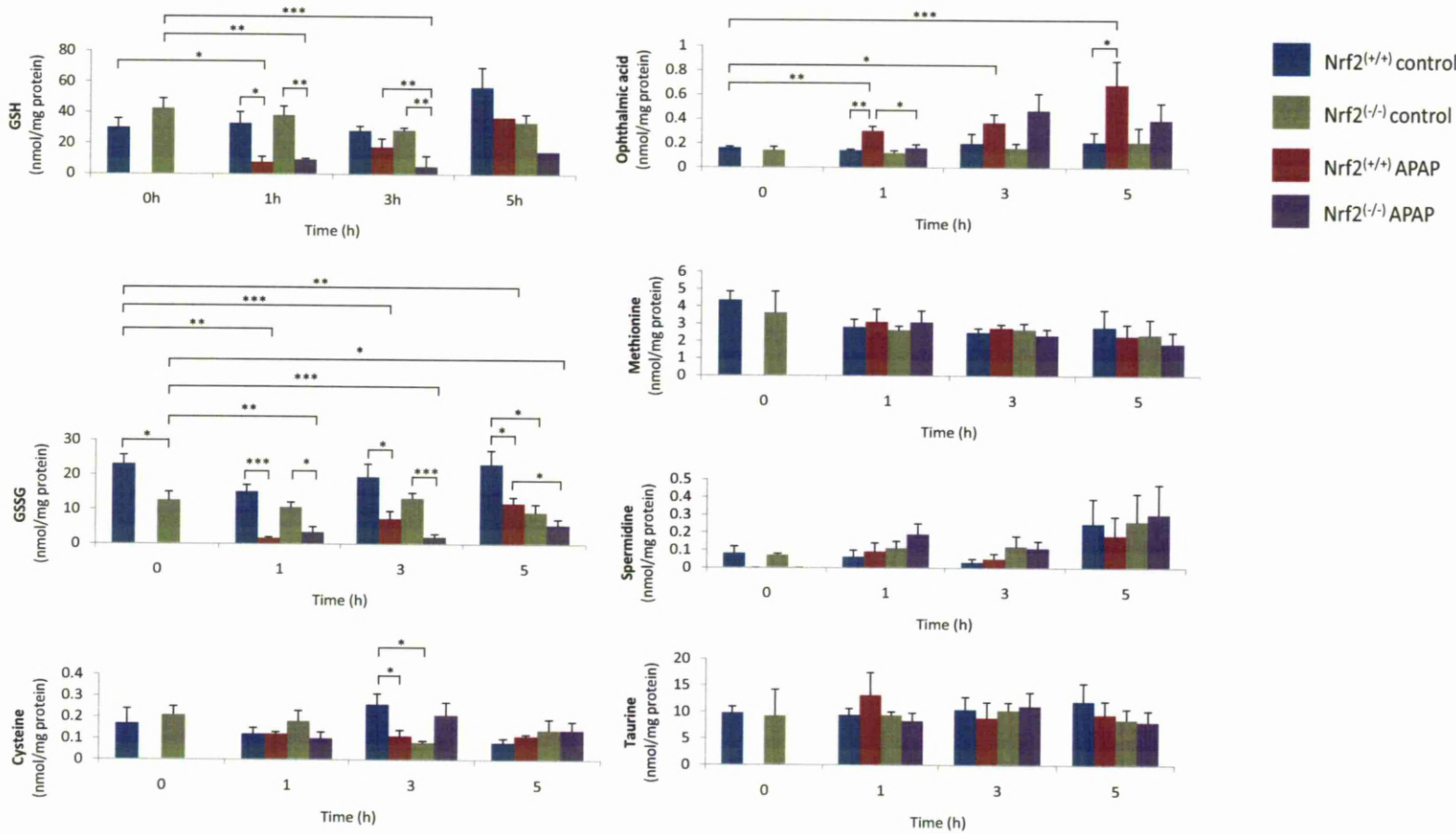


Figure 3.4 Hepatic metabolite profile of $Nrf2^{(+/+)}$ and $Nrf2^{(-/-)}$ mice after treatment with an acute dose of APAP. Male C57BL/6 mice were administered an acute dose of 350 mg/kg APAP or saline for 0, 1, 3 and 5 h. Liver samples were collected from each treatment groups per time-point and processed for LC-MS-MS metabolite profiling as detailed in sections 3.2.3 and 3.2.9 for the determination of cysteine, ophthalmic acid, GSSG, taurine, spermidine and methionine content. Data analysis was carried out using Analyst QS 2.0. GSH was determined according to the protocol of Vandeputte *et al.* (1994) as detailed in section 3.2.7. Results are presented as mean nmol/mg protein (± SEM), n = 4-6. Asterisks(*) represent a statistical difference (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) compared with time-matched control.

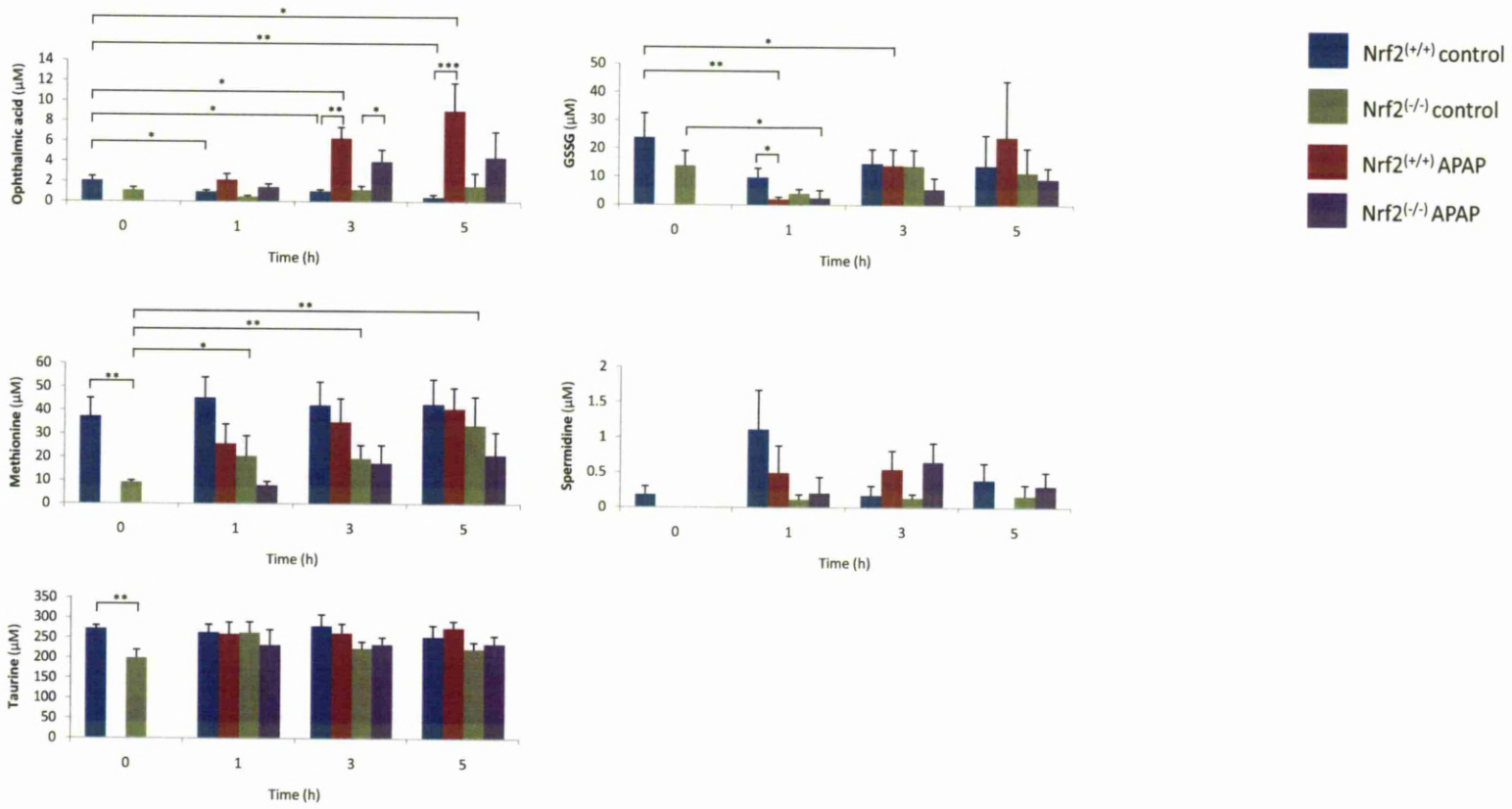


Figure 3.5 Metabolite profile in the serum of $Nrf2^{(+/+)}$ and $Nrf2^{(-/-)}$ mice after treatment with an acute dose of APAP. Male C57BL/6 mice were administered an acute dose of 350 mg/kg APAP or saline for 0, 1, 3 and 5 h. Serum samples were collected from each treatment groups per time-point and processed for LC-MS-MS metabolite profiling as detailed in sections 3.2.3 and 3.2.9 for the determination of ophthalmic acid, GSSG, taurine, spermidine and methionine content. Data analysis was carried out using Analyst QS 2.0. Results are presented as mean μM (± SEM) n = 4-6. Asterisks (*) represent a statistical difference (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) compared with time-matched control.

3.3.2 Assessing metabolite perturbations in the GSH system upon chronic treatment of $Nrf2^{(+/+)}$ and $Nrf2^{(-/-)}$ mice with escalating doses of APAP (150 – 600 mg/kg)

$Nrf2^{(+/+)}$ and $Nrf2^{(-/-)}$ were administered escalating doses (150 – 600 mg/kg) of APAP for 8 days as illustrated in Table 3.2 and described in section 3.2.4. All samples were analysed for the selected metabolites within the GSH system as described in section 3.2.9. Serum ALT levels were also measured as detailed in section 3.2.6 to indicate liver injury and/or toxicity as a consequence of chronic paracetamol treatment as described above.

3.3.2.1 Determination of hepatic injury by serum alanine transferase measurement

Minimal increase in serum ALT was observed in the $Nrf2^{(+/+)}$ APAP-treated group (51.13 ± 8.05 U/L) compared with control (17.66 ± 1.99 U/L) (Figure 3.6). In contrast, the $Nrf2^{(-/-)}$ APAP-treated animals had a mean ALT of 721.21 ± 373.33 U/L compared with 41.95 ± 12.91 U/L in the corresponding controls. These data show that chronic treatment of $Nrf2^{(+/+)}$ mice with escalating doses of APAP ranging from sub-toxic to toxic levels results in a marked ability to withstand what would represent a toxic challenge of APAP in acutely challenged mice. $Nrf2^{(-/-)}$ mice, whilst showing a reduced hepatotoxic response compared with an untreated mouse did nevertheless, show enhanced liver injury/toxicity as assessed by ALT compared with the wild type.

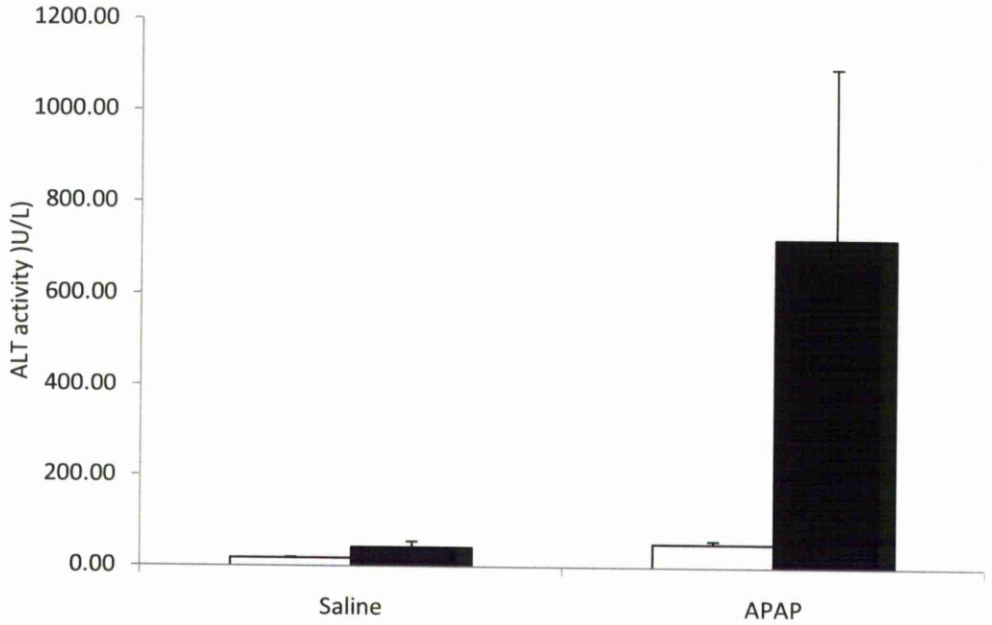


Figure 3.6 Serum ALT activity in mice after chronic treatment with escalating doses of APAP. Male C57BL/6 $Nrf2^{(+/+)}$ (□) and $Nrf2^{(-/-)}$ (■) mice were administered with escalating doses (150 – 600 mg/kg) of APAP or saline over an 8 day period (3.2.4). Blood samples were collected and analysed for ALT activity as detailed in 3.2.6. Data are presented as mean serum ALT activity (U/L) (± SEM), n = 4 animals.

3.3.2.2 Metabolite profile of $Nrf2^{(+/+)}$ and $Nrf2^{(-/-)}$ mice in response to chronic treatment with escalating doses of APAP

Glutathione and metabolites (GSSG, methionine, cysteine, taurine, spermidine and ophthalmic acid) associated with the glutathione biosynthetic pathway were measured in the liver and serum of $Nrf2^{(+/+)}$ and $Nrf2^{(-/-)}$ mice administered with escalating doses of APAP or saline (control) for 8 consecutive days to determine perturbations within the GSH system and whether these were or were not Nrf2-mediated. Figures 3.7 (hepatic) and 3.8 (serum) represent the effect of APAP on the GSH system under the treatment described above in the $Nrf2^{(+/+)}$ and $Nrf2^{(-/-)}$mice.

A treatment regimen of increasing APAP dose had no significant effect on the majority of metabolites measured in the liver and serum between APAP and saline-treated mice of both strains.

Numerically, hepatic GSH in the $Nrf2^{(+/+)}$ control animals was higher (70.9 ± 4.90 nmol/mg protein) compared with the $Nrf2^{(-/-)}$ control group (52.5 ± 4.50 nmol/mg protein). Interestingly, an increase in hepatic GSH was observed in both $Nrf2^{(+/+)}$ and $Nrf2^{(-/-)}$ APAP-treated animals (81.20 ± 3.30 and 80.00 ± 12.80 nmol/mg protein) compared with their respective controls but this difference was more prominent in the $Nrf2^{(-/-)}$ animals.

No difference in hepatic ophthalmic acid was observed between the APAP (0.60 ± 0.28 nmol/mg protein) and saline-treated (0.63 ± 0.36 nmol/mg protein) $Nrf2^{(+/+)}$ animals. However, although not determined to be significant, a marked increase in hepatic ophthalmic acid was observed in the APAP-treated $Nrf2^{(-/-)}$ mice (0.98 ± 0.34 nmol/mg protein) compared with control (0.16 ± 0.03 nmol/mg protein). Interestingly, hepatic ophthalmic acid was higher in the saline-treated $Nrf2^{(+/+)}$ animals compared with their $Nrf2^{(-/-)}$ counterparts, whilst the opposite was observed in the APAP-treated groups.

Serum levels of ophthalmic acid were lower in the APAP-treated animals of both strains (1.49 ± 0.43 and 0.95 ± 0.21 μM for $Nrf2^{(+/+)}$ and $Nrf2^{(-/-)}$ respectively) compared with their respective controls (3.71 ± 2.02 and 1.21 ± 0.53 μM for $Nrf2^{(+/+)}$ and $Nrf2^{(-/-)}$ respectively) but were not determined to be statistically significant. Serum ophthalmic acid in the $Nrf2^{(-/-)}$ animals of both treatment groups were consistently lower when compared with their wild type counterparts.

Levels of hepatic GSSG were lower in the $Nrf2^{(+/+)}$ APAP-treated animals (44.63 ± 13.77 nmol/mg protein) compared with the control group (76.74 ± 17.16 nmol/mg protein). In contrast, an increase in hepatic GSSG was observed in the $Nrf2^{(-/-)}$ APAP-treated animals (78.97 ± 22.00 nmol/mg protein) compared with control (45.73 ± 2.07 nmol/mg protein). When comparing between $Nrf2^{(+/+)}$ and $Nrf2^{(-/-)}$, hepatic GSSG was lower in the $Nrf2^{(-/-)}$ saline-treated animals whilst levels were higher in the $Nrf2^{(-/-)}$ APAP-treated groups in comparison to their wild type counterparts.

Interestingly, serum GSSG was lower in the APAP-treated groups of both strains (10.45 ± 3.79 and 2.77 ± 1.24 μM for $Nrf2^{(+/+)}$ and $Nrf2^{(-/-)}$ respectively) compared with their respective controls (18.86 ± 18.86 and 26.39 ± 17.02 μM for $Nrf2^{(+/+)}$ and $Nrf2^{(-/-)}$ respectively). When comparing between $Nrf2^{(+/+)}$ and $Nrf2^{(-/-)}$, serum GSSG was higher in the $Nrf2^{(-/-)}$ saline-treated animals whilst levels were lower in the $Nrf2^{(-/-)}$ APAP-treated groups in comparison with their wild type counterparts.

Hepatic spermidine was lower in both APAP-treated strains (0.02 ± 0.01 nmol/mg protein for both $Nrf2^{(+/+)}$ and $Nrf2^{(-/-)}$) compared with their respective controls (0.09 ± 0.08 and 0.08 ± 0.06 nmol/mg protein for $Nrf2^{(+/+)}$ and $Nrf2^{(-/-)}$ respectively). No notable differences were observed in hepatic and serum spermidine levels between the two strains in response to either saline or APAP treatment.

No notable differences were observed in hepatic methionine of both strains under the two treatment groups. However, $Nrf2^{(+/+)}$ APAP-treated animals demonstrated a significantly lower level of serum methionine (37.54 ± 2.45 μM) compared with control (52.71 ± 3.33 μM). Serum methionine in $Nrf2^{(-/-)}$ saline-treated animals were also significantly lower (43 ± 1.96 μM) compared with their wild type counterparts (52.71 μM). In contrast, a higher level of serum methionine was measured from $Nrf2^{(-/-)}$ APAP-treated animals (47.46 ± 6.85 μM) compared with its wild type counterpart (37.54 ± 2.45 μM) .

A significant increase in hepatic taurine was observed in the $Nrf2^{(-/-)}$ APAP-treated animals (27.79 ± 1.46 nmol/mg protein) compared with the control group (19.51 ± 2.38 nmol/mg protein), and was also higher when compared with the $Nrf2^{(+/+)}$ APAP-treated group (17.14 ± 2.45 nmol/mg protein). No other notable changes in liver and serum taurine were observed.

Hepatic cysteine was determined to be higher in the $Nrf2^{(+/+)}$ APAP-treated animals (0.32 ± 0.11 nmol/mg protein) compared with control (0.18 ± 0.03 nmol/mg protein) and levels of hepatic cysteine in the wild type were in general higher under both treatments when compared with the THLE-Nulls (0.14 ± 0.03 and 0.17 ± 0.01 nmol/mg protein for $Nrf2^{(-/-)}$ saline and APAP-treated animals respectively).

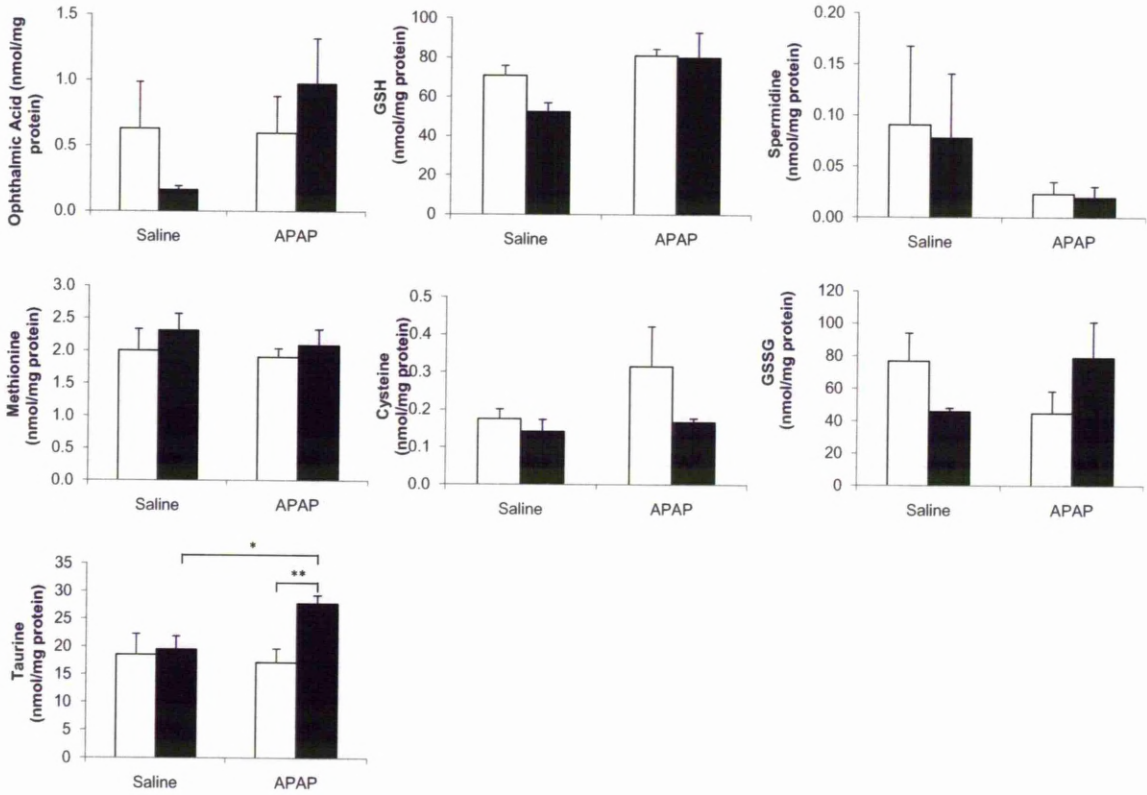


Figure 3.7 Hepatic metabolite profile of Nrf2$^{(+/+)}$ (□) and Nrf2$^{(-/-)}$ (■)mice after a chronic treatment with escalating doses of APAP. Male C57BL/6 mice were administered with escalating doses of APAP (150-600 mg/kg) for 8 days. Livers were isolated from each treatment group and processed for LC-MS-MS metabolite profiling as detailed in sections 3.2.3 and 3.2.9 for the determination of cysteine, ophthalmic acid, GSSG, taurine, spermidine and methionine content. Data analysis was carried out using Analyst QS 2.0. GSH was determined according to the method of Vandeputte *et al.,* (1994) as detailed in section 3.2.7. Results are presented as mean nmol/mg protein (± SEM) n = 4. Asterisks(*) represent a statistical difference (*p < 0.05, **p < 0.01, ***p < 0.001) compared with time-matched control.

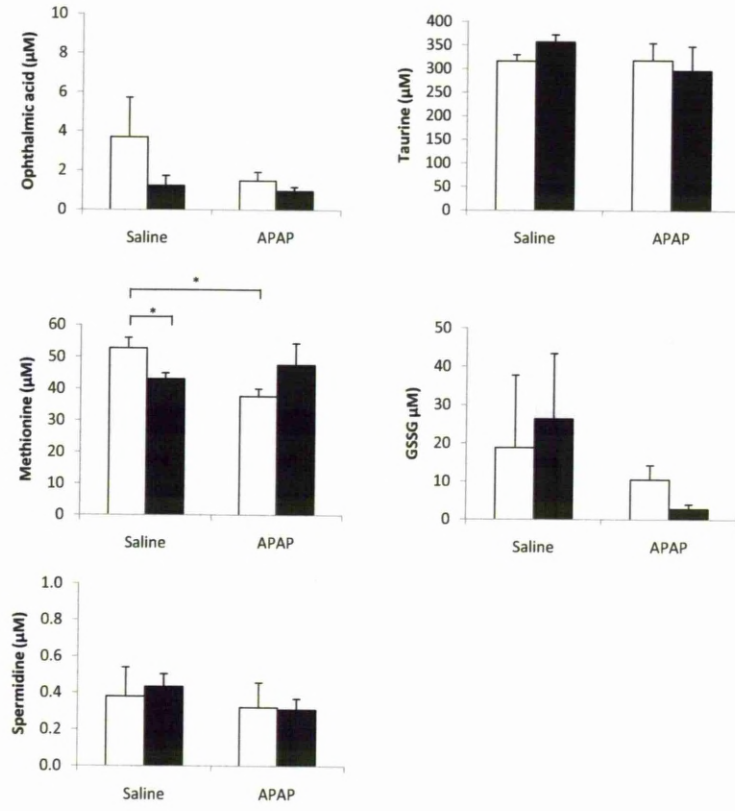


Figure 3.8 Metabolite profile in the serum of $Nrf2^{(+/+)}$ (□)and $Nrf2^{(-/-)}$ (■)mice after a chronic treatment with escalating doses of APAP. Male C57BL/6 $Nrf2^{(+/+)}$ mice were administered with escalating doses of APAP (150-600 mg/kg) for 8 days. Blood samples were collected and processed for LC-MS-MS metabolite profiling as detailed in sections 3.2.3 and 3.2.9 for the determination of ophthalmic acid, GSSG, taurine, spermidine and methionine content. Data analysis was carried out using Analyst QS 2.0. Results are presented as mean µM (± SEM), n = 3-4. Asterisks(*) represent a statistical difference (*p < 0.05, **p < 0.01, ***p < 0.001) compared with time-matched control.

3.3.3 Metabolite perturbations in the GSH system upon chronic treatment of $Nrf2^{(+/+)}$ and $Nrf2^{(-/-)}$ mice with escalating doses of APAP (150 – 600 mg/kg) for 8 days followed by a lethal dose of 1000 mg/kg APAP on day 9

The ability of mice to withstand a hepatotoxic dose of APAP following pretreatment with increasing doses (150 – 600 mg/kg) over an 8-day treatment regimen (Table 3.2) was assessed in both $Nrf2^{(+/+)}$ and $Nrf2^{(-/-)}$ animals by administration with a final toxic dose challenge of 1000 mg/kg APAP on day 9. A summary of all the treatment groups during this investigation is summarised in Table 3.4.

Table 3.4 Abbreviations used to denote the different treatment groups:

Abbreviation	Dosing regimen
SS	Mice dosed with 0.9 % saline for 9 consecutive days.
SA	Mice treated with 0.9 % saline for 8 consecutive days followed by a lethal dose of 1000 mg/kg APAP on day 9.
AS	Mice treated with escalating doses of APAP for 8 consecutive days followed by a dose of 0.9 % saline on day 9.
AA	Mice treated with escalating doses of APAP for 8 consecutive days followed by a lethal dose of 1000 mg/kg APAP on day 9.

3.3.3.1 Determination of hepatic injury by measurement of serum ALT

Serum ALT was measured to determine whether chronic treatment with escalating doses of APAP can provide protection from a subsequent toxic dose in mice and whether the deletion of the Nrf2 gene has made the $Nrf2^{(-/-)}$ animals more susceptible to APAP-mediated toxicity compared with $Nrf2^{(+/+)}$.

Following the 1000 mg/kg challenge dose, serum ALT in the $Nrf2^{(+/+)}$SA group was significantly higher (3263.2 ± 823.49 U/L) compared with the control group, $Nrf2^{(+/+)}$SS (25.64 ± 5.31 U/L) (Figure 3.9-A). In the wild type mice pretreated with APAP ($Nrf2^{(+/+)}$AA), a smaller though significant increase in serum ALT was also observed (993.84 ± 317.85 U/L) compared with its corresponding control group, $Nrf2^{(+/+)}$AS (141.98 ± 79.17 U/L). Although

both $Nrf2^{(+/+)}$SA and $Nrf2^{(+/+)}$AA had significantly higher serum ALT compared with their respective controls, the $Nrf2^{(+/+)}$SA group was more susceptible to APAP-induced hepatic injury compared with the $Nrf2^{(+/+)}$AA group. Furthermore, these data suggest that pretreatment with escalating doses of APAP may have afforded the wild type animals protection against a toxic dose challenge.

Although a similar pattern in serum ALT to the wild type was also observed in the $Nrf2^{(-/-)}$ animals (Figure 3.9-A), serum ALT levels measured in the $Nrf2^{(-/-)}$ animals were only from those that survived. As shown by the survival data in Figure 3.9-B, chronic treatment with saline followed by a toxic dose challenge of APAP (SA) was 100 % lethal to the $Nrf2^{(-/-)}$ animals. This was also demonstrated in the $Nrf2^{(-/-)}$ animals that were pretreated and subsequently challenged with APAP ($Nrf2^{(-/-)}$AA), although the number of deaths in this group was marginally smaller compared with the $Nrf2^{(-/-)}$SA group. The difference observed between these two groups suggests an important role of Nrf2 in the adaptive response of mice to chemical stress.

Measurement of serum ALT revealed a significant increase in the $Nrf2^{(-/-)}$SA group (1484.62 ± 435.46 U/L) compared with the $Nrf2^{(-/-)}$SS group (18.52 ± 2.45 U/L). Similarly, although not significant, serum ALT in the $Nrf2^{(-/-)}$AA group (468.09 ± 318.96 U/L) was higher compared with control, $Nrf2^{(-/-)}$AS (153.31 ± 64.13 U/L). The serum ALT data in the $Nrf2^{(-/-)}$ animals suggest that the saline pretreated group challenged with a lethal dose of APAP (SA) was more susceptible to APAP-induced toxicity in comparison to the APAP pretreated group (AA). Furthermore, based on a clinical chemistry endpoint, as this data was consistent with that observed in the $Nrf2^{(+/+)}$ animals, the absence of the Nrf2 gene in the $Nrf2^{(-/-)}$ animals did not enhance the susceptibility of the $Nrf2^{(-/-)}$ animals to APAP-induced toxicity. Observations in the serum ALT of both the $Nrf2^{(+/+)}$ and $Nrf2^{(-/-)}$ animals were therefore APAP-mediated.

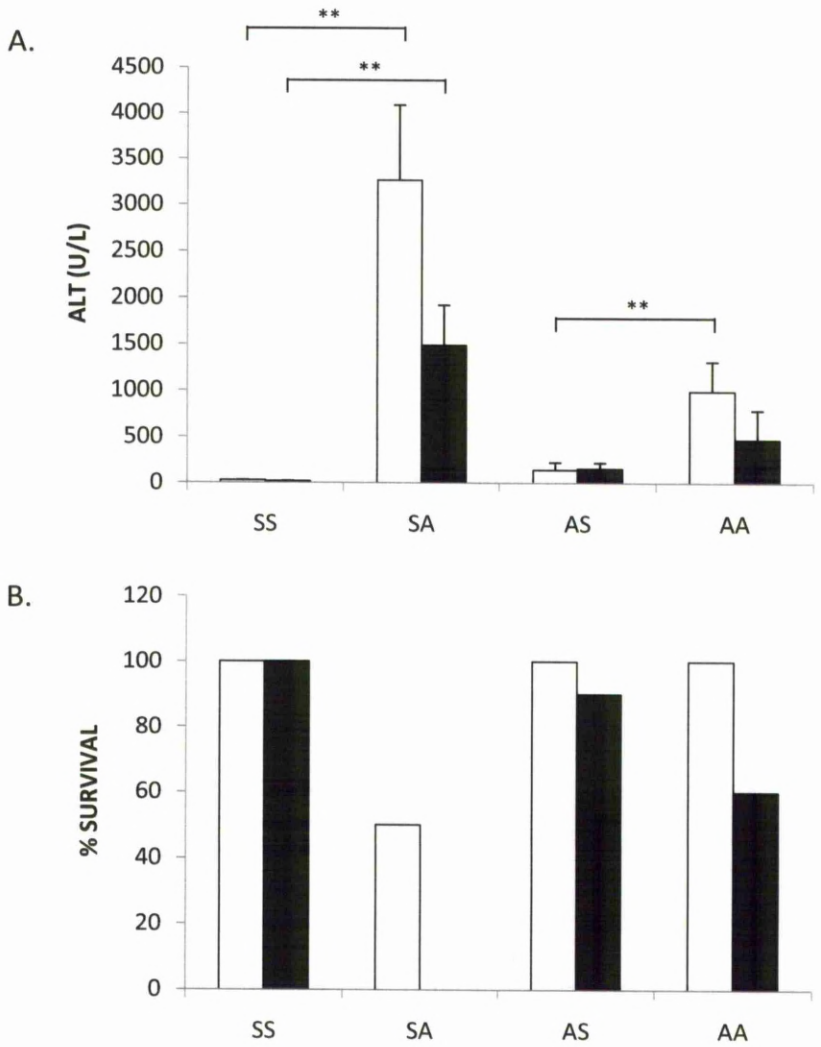


Figure 3.9 Serum ALT activity and survival rate in mice pretreated with escalating doses of APAP and subsequently challenged with a toxic dose. Male C57BL/6 $Nrf2^{(+/+)}$ (□) and $Nrf2^{(-/-)}$ (■) mice were administered with escalating doses of APAP (150 – 600 mg/kg) or saline over an 8 day period and then challenged with a 1000 mg/kg APAP or saline on day 9 for 5 h. Blood samples were collected and analysed for serum ALT activity (A) as detailed in 3.2.6 and survival rate across the duration of the treatment (B). Data are presented as mean serum ALT activity (U/L) (± SEM) or percentage of survival rate, n = 4-8 animals).

3.3.3.2 Metabolite analysis by LC-MS-MS

Figures 3.10 (hepatic) and 3.11 (serum) represent the effect of APAP treatment at escalating doses for 8 days and then challenged with a toxic dose on day 9 on the GSH system in the liver and serum of the $Nrf2^{(+/+)}$ and $Nrf2^{(-/-)}$mice.

3.3.3.2.1 Comparison between $Nrf2^{(+/+}$SS, $Nrf2^{(-/-)}$SS, $Nrf2^{(+/+)}$SA and $Nrf2^{(-/-)}$SA treatment groups

Mean GSH, GSSG and methionine levels were significantly lower in the livers of both $Nrf2^{(+/+)}$SA (2.98 ± 0.77, 1.01 ± 0.11 and 0.86 ± 0.07 nmol/mg protein respectively) and $Nrf2^{(-/-)}$SA (3.07 ± 0.84, 1.27 ± 0.18 and 1.05 ± 0.18 nmol/mg protein) in comparison to levels observed in their respective controls, $Nrf2^{(+/+)}$SS (74.62 ± 2.04, 57.59 ± 15.35 and 1.81 ± 0.13 nmol/mg protein respectively) and $Nrf2^{(-/-)}$SS (37.46 ± 1.89, 34.69 ± 5.18 and 2.37 ± 0.17 nmol/mg protein) groups.

In contrast, mean hepatic ophthalmic acid and cysteine levels within the $Nrf2^{(+/+)}$SA groups (0.58 ± 0.14 and 0.12 ± 0.02 nmol/mg protein respectively) were significantly higher in comparison to the $Nrf2^{(+/+)}$SS group (0.11 ± 0.02 and 0.05 ± 0.00 nmol/mg protein respectively). This was also observed in the equivalent $Nrf2^{(-/-)}$ animals where hepatic ophthalmic acid and cysteine were determined to be 0.68 ± 0.11 and 0.14 ± 0.02 nmol/mg protein respectively in the $Nrf2^{(-/-)}$SA animals compared with 0.063 ± 0.02 and 0.023 ± 0.00 nmol/mg protein respectively in the $Nrf2^{(-/-)}$SS animals but only the difference in ophthalmic acid was determined to be significant. A noticeably higher level of hepatic spermidine was also observed in both the $Nrf2^{(+/+)}$SA and $Nrf2^{(-/-)}$SA groups (0.07 ± 0.04 and 0.18 ± 0.11 nmol/mg protein respectively) in comparison to control (0.02 ± 0.01 nmol/mg protein for both strains) but were also not determined to be significant.

Similar to that observed in the liver, significantly higher ophthalmic acid (3.10 ± 0.46 and 2.29 ± 0.33 μM respectively) was also observed in the serum of both $Nrf2^{(+/+)}$SA and $Nrf2^{(-/-)}$SA groups in comparison to their respective controls (0.51 ± 0.08 and 0.26 ± 0.05 μM for $Nrf2^{(+/+)}$SS and $Nrf2^{(-/-)}$SS respectively). Consistent with hepatic levels, serum GSSG was also lower in the $Nrf2^{(+/+)}$SA group (0.25 ±0.25 μM) compared with control (15.33 ± 4.03 μM for $Nrf2^{(+/+)}$SS). Furthermore, no serum GSSG was detected in the $Nrf2^{(-/-)}$SA, whilst 7.81 ± 3.12 μM was measured in the serum of the control group $Nrf2^{(-/-)}$SS. Spermidine in the

serum was also higher in the $Nrf2^{(+/+)}$SA (1.3 ± 0.32 μM) and $Nrf2^{(-/-)}$SA (1.21 ± 0.47 μM) groups compared with their respective controls (0.41 ± 0.12 and 0.72 ± 0.43 μM for $Nrf2^{(+/+)}$SS and $Nrf2^{(-/-)}$SS respectively). Methionine on the other hand only demonstrated a significant difference in the $Nrf2^{(-/-)}$ animals where it was lower in the $Nrf2^{(-/-)}$SA animals (31.14 ± 4.51 μM) compared with the $Nrf2^{(-/-)}$SS group (51.40 ± 2.13 μM).

Although no notable change was observed in hepatic taurine levels in both SA and SS treatment groups of both strains, taurine measured in the serum of $Nrf2^{(+/+)}$SA (251.72 ± 20.71 μM) and $Nrf2^{(-/-)}$SA (228.42 ± 16.39 μM) animals were significantly lower compared with their respective controls (336.93 ± 22.72 and 331.45 ± 6.69 μM for $Nrf2^{(+/+)}$SS and $Nrf2^{(-/-)}$SS respectively).

3.3.3.2.2 Comparison between $Nrf2^{(+/+)}$AS, $Nrf2^{(-/-)}$AS , $Nrf2^{(+/+)}$AA and $Nrf2^{(-/-)}$AA

Significantly lower levels of GSH (4.23 ± 1.09 and 8.06 ± 2.54 nmol/mg protein), GSSG (1.74 ± 0.49 and 5.00 ± 3.02 nmol/mg protein), as well as a marked but not significant decrease in methionine (0.93 ± 0.00 and 0.74 ± 0.11 nmol/mg protein) and cysteine (0.08 ± 0.02 and 0.15 ± 0.04 nmol/mg protein) were observed in the liver of $Nrf2^{(+/+)}$AA and $Nrf2^{(-/-)}$AA groups respectively, in comparison to their corresponding controls, $Nrf2^{(+/+)}$AS (44.02 ± 12.06, 25.08 ± 2.63, 1.83 and 0.19 ± 0.07 nmol/mg protein respectively) and $Nrf2^{(-/-)}$AS (35.01 ± 8.16, 19.03 ± 1.38, 2.24 ± 0.44 and 0.19 ± 0.02 nmol/mg protein). Interestingly, hepatic methionine was significantly higher in the $Nrf2^{(-/-)}$AS group (2.24 ± 0.44 nmol/mg protein) compared with its wild type counterpart (1.83 ± 0.20 nmol/mg protein)

Consistent with that observed in the liver, GSSG levels in the serum of the $Nrf2^{(+/+)}$AA group (0.22 ± 0.22 μM respectively) was significantly lower in comparison to the $Nrf2^{(+/+)}$AS group (20.32 ± 6.14 μM). GSSG in the serum of $Nrf2^{(-/-)}$AA group was not detected whilst 3.14 ± 2.06 μM was observed in the control ($Nrf2^{(-/-)}$AS). This was in agreement with that observed in the liver although to a lesser extent as hepatic GSSG were at detectable levels in all treatment groups.

On the other hand, ophthalmic acid in the liver and serum of the $Nrf2^{(+/+)}$AA and $Nrf2^{(-/-)}$AA groups (2.81 ± 0.38 and 2.86 ± 0.79 nmol/mg protein in the liver and 18.99 ± 7.47 and 6.03 ± 1.03 μM in the serum) were significantly higher in comparison to the $Nrf2^{(+/+)}$AS and

$Nrf2^{(-/-)}$AS groups (0.28 ± 0.09 and 0.21 ± 0.06 nmol/mg protein in the liver and 1.02 ± 0.22 and 0.45 ± 0.13 μM in the serum), consistent with the GSH depletion observed from the same animals.

Hepatic spermidine in the $Nrf2^{(-/-)}$AA animals (0.03 ± 0.00 nmol/mg protein) was significantly lower compared with the control group, $Nrf2^{(-/-)}$AS (0.09 ± 0.04 nmol/mg protein). Similar to the saline pretreated animals, higher levels of hepatic spermidine in the $Nrf2^{(+/+)}$AA animals (0.04 ± 0.01 nmol/mg protein) were detected in comparison to the $Nrf2^{(+/+)}$AS group (0.02 ± 0.01 nmol/mg protein). This was in contrast to what was observed in the serum where spermidine in the $Nrf2^{(+/+)}$AA group was lower (0.15 ± 0.10 μM) compared with the control $Nrf2^{(+/+)}$AS animals (0.51 ± 0.15 μM).

In contrast to the unaltered levels of hepatic taurine, a significant decrease was observed in the serum of $Nrf2^{(+/+)}$AA and $Nrf2^{(-/-)}$AA groups (174.48 ± 15.44 and 170.97 ± 9.11 μM respectively) compared with the control groups (265.05 ± 12.55 and 209.75 ± 9.64 μM), consistent with that observed in the serum of the saline pretreated animals. Furthermore, taurine levels in the serum of the $Nrf2^{(-/-)}$AS animals were significantly lower compared with its wild type counterpart.

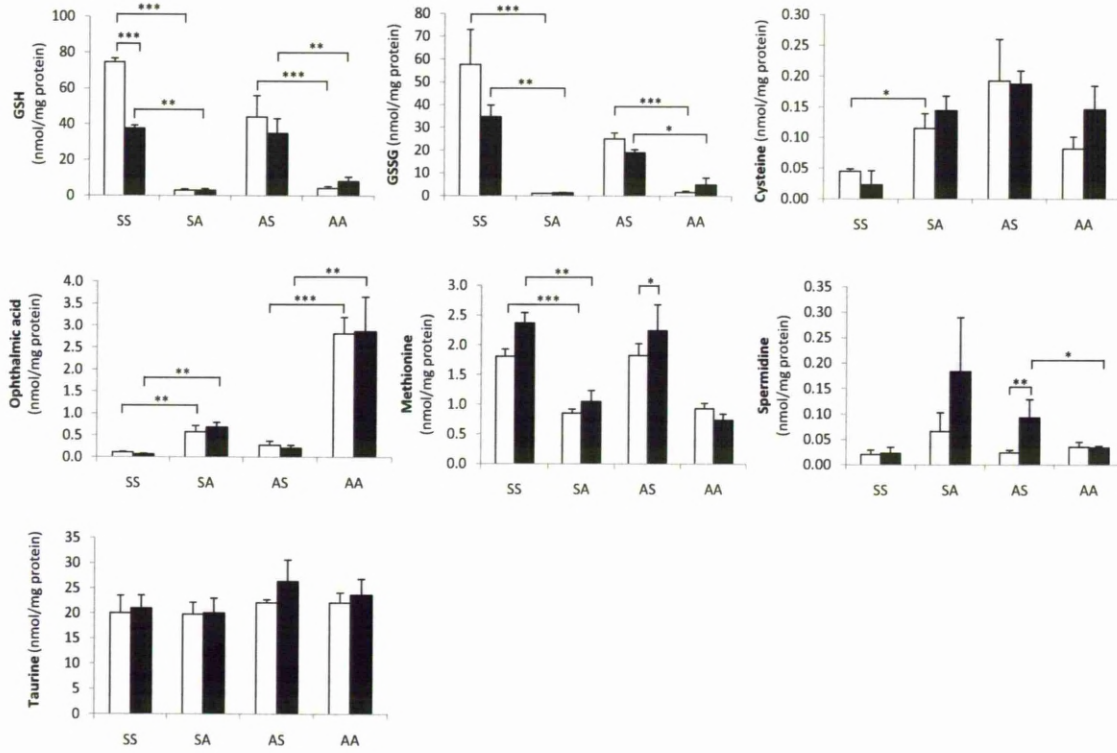


Figure 3.10 Hepatic metabolite profile of Nrf2$^{(+/+)}$ (□) and Nrf2$^{(+/+)}$ (■) mice pretreated with escalating doses of APAP and subsequently challenged with a toxic dose. Male C57BL/6 Nrf2$^{(+/+)}$ mice were administered with escalating doses of APAP (150 – 600 mg/kg) for 8 days followed by 1000 mg/kg APAP on day 9 for 5 h. Livers were isolated and processed for LC-MS-MS metabolite profiling as detailed in sections 3.2.3 and 3.2.9 for the determination of cysteine, ophthalmic acid, GSSG, taurine, spermidine and methionine content. Data analysis was carried out using Analyst QS 2.0. GSH was determined according to the method of Vandeputte *et al.*, (1994) as detailed in section 3.2.7. Results are presented as mean nmol/mg protein (± SEM), n = 4 -8. Asterisks (*) represent a statistical difference (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) compared with time-matched control.

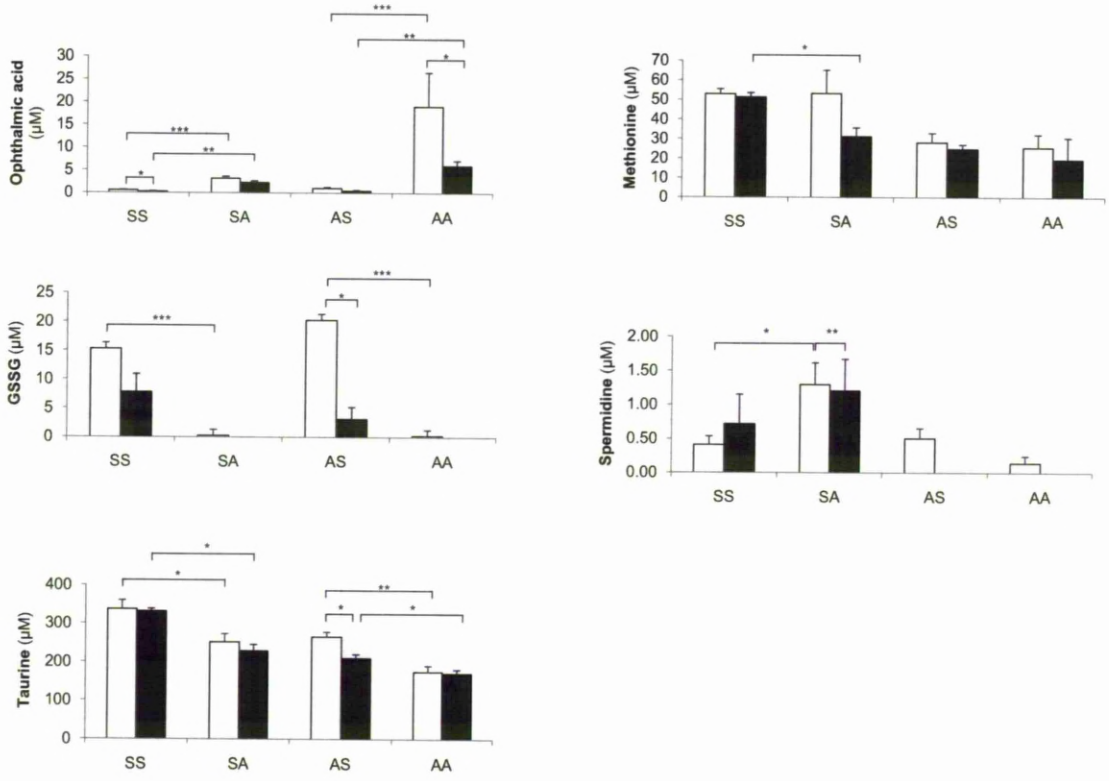


Figure 3.11 Metabolite profile in the serum of $Nrf2^{(+/+)}$ (□) and $Nrf2^{(-/-)}$ (■) mice after pretreatment with escalating doses of APAP and subsequently challenged with a toxic dose. Male C57BL/6 $Nrf2^{(+/+)}$ mice were administered with escalating doses of APAP (150-600 mg/kg) for 8 days followed by 1000 mg/kg APAP on day 9 for 5 h. Blood samples were collected and processed for LC-MS-MS metabolite profiling as detailed in sections 3.2.3 and 3.2.9 for the determination of ophthalmic acid, GSSG, taurine, spermidine and methionine content. Data analysis was carried out using Analyst QS 2.0. Results are presented as mean μM (± SEM) n = 4 -8. Asterisks (*) represent a statistical difference (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) compared with time-matched control.

3.4 DISCUSSION

The purpose of the studies described in this chapter was to investigate the utility of the $Nrf2^{(-/-)}$ mouse as a model of chemical stress within the context of the GSH system. All studies were carried out using the $Nrf2^{(+/+)}$ mouse as a comparator and APAP as the chemical stress-inducer. To address the role of Nrf2 in the immediate response of the mouse to chemical stress, both $Nrf2^{(+/+)}$ and $Nrf2^{(-/-)}$ were administered an acute sub-toxic dose of APAP over a 5 h period. Serum ALT as an indicator of liver injury/toxicity and selected metabolites that represent pathways in the GSH system as detailed in chapter 1 were measured. Further to this, the role of Nrf2 in the adaptive response of the mouse to chemical stress was also investigated through chronic dosing with escalating doses (0-600 mg/kg) of APAP as a means of preconditioning these animals to an APAP-mediated environment. To gain insight into modulations within the GSH system if any, as one potential mechanism of adaptation to chronic APAP treatment and to determine whether this treatment was toxic, the same parameters to those measured in the acute study were also taken at the end of the 8-day APAP treatment described here. To determine if this preconditioning period has provided protection against a subsequent toxic dose challenge of APAP, another set of $Nrf2^{(+/+)}$ and $Nrf2^{(-/-)}$ mice were also preconditioned with escalating doses of APAP for 8 days as above and were this time challenged with a toxic dose of 1000 mg/kg APAP on day 9. The same parameters as stated above were also measured in these animals.

3.4.1 Acute time-course study with 350 mg/kg APAP

As mentioned above, the aim of this experiment was to elucidate the role of Nrf2 in the immediate response of the mouse within the context of the GSH system after exposure to an acute sub-toxic dose (350 mg/kg) of APAP. The significant increases in serum ALT at 3 and 5 h indicated liver injury as a consequence of APAP-treatment in both $Nrf2^{(+/+)}$ and $Nrf2^{(-/-)}$ mice (Figure 3.3). At closer inspection, the effect of this treatment was the same to both strains at 3 h, but only progressed to a more notable increase in serum ALT in the $Nrf2^{(-/-)}$ animals at 5 h whilst levels remained the same in the $Nrf2^{(+/+)}$ demonstrating a higher susceptibility of the $Nrf2^{(-/-)}$ mice to APAP-induced toxicity at doses that are tolerated by their wild type counterparts. This is consistent with findings by other groups who have also shown enhanced susceptibility of Nrf2 deficient mice to APAP-induced toxicity (Chan *et*

al., 2001, Enomoto *et al.*, 2001) and to other hepatotoxicants including alcohol (Lamle *et al.*, 2008), pentachlorophenol (Umemura *et al.*, 2006), carbon tetrachloride (Xu *et al.*, 2008) and arsenic (Jiang *et al.*, 2009). The attenuation of further liver injury in the APAP-treated $Nrf2^{(+/+)}$ mice may be an indication of an adaptive response induced in the wild type mice therefore providing protection . On the other hand, this ability to adapt to APAP-induced stress may have been abrogated in the $Nrf2^{(-/-)}$ mice as a consequence of Nrf2 deletion resulting in a further increase in serum ALT at 5 h.

The differences in serum ALT levels between the $Nrf2^{(-/-)}$ and $Nrf2^{(+/+)}$ mice are supported by the trend in hepatic GSH levels observed in the same animals (Figures 3.4 and 3.5). Immediate significant depletion of hepatic GSH at 1 h was observed in both strains, indicative of the overwhelming level of APAP-induced chemical stress as a consequence of APAP bioactivation (Mitchell *et al.*, 1973a). However, this was followed by a consistent time-dependent increase in hepatic GSH in the $Nrf2^{(+/+)}$ APAP-treated animals where levels were almost comparable to the control group at 5 h. On the other hand, an attempt to restore hepatic GSH to normal levels by the $Nrf2^{(-/-)}$ APAP-treated animals was only observed at 5 h where the difference in GSH levels between the $Nrf2^{(+/+)}$ and $Nrf2^{(-/-)}$ animals was not determined to be significant, a contrast to what was observed at 1 and 3 h. This may in part explain the difference in serum ALT observed between the two strains, as the inability of the $Nrf2^{(-/-)}$ animals to up-regulate GSH synthesis readily may have led to continued liver damage and therefore leakage of hepatic ALT into the circulation. On the other hand, the ability of the $Nrf2^{(+/+)}$ animals to up-regulate GSH synthesis more readily may have allowed the detoxification of reactive species and restore redox balance much quicker. Liver damage was therefore less severe and hence no further increase in serum ALT was seen at 5 h. As such, Nrf2 up-regulation of GSH synthesis (Chan and Kwong, 2000, Chan *et al.*, 2001, Enomoto *et al.*, 2001) plays an important role in the response to acute chemical stress as demonstrated by the GSH data presented here. The decrease in hepatic GSH seen following APAP treatment was mirrored by levels of GSSG in the serum and liver of both strains potentially as a consequence of the GSH redox cycling pathway as described in chapter 1, indicating the lack of GSH available for this process as a consequence of APAP treatment.

Increasing levels of ophthalmic acid in the liver and serum were observed in both strains in response to APAP treatment, however in comparison to their respective controls, this trend was more prominent in the $Nrf2^{(+/+)}$ animals compared with the $Nrf2^{(-/-)}$ animals (Figure 3.4). This is consistent in terms of the similarities in the synthesis of GSH and

ophthalmic acid (Chapter 1, Figure 1.8), both of which share the same enzymes and precursors, apart from cysteine which is used in GSH synthesis and is replaced with 2-AB in the synthesis of ophthalmic acid. Although GCL, the enzyme responsible for the rate limiting step of GSH synthesis, has a higher affinity for cysteine (Km reported as 0.3 mM, Seelig and Meister, 1985) than 2-AB (Km reported as 1.0 mM, Seelig and Meister, 1985), the switch in GCL activity to ophthalmic acid synthesis is clearly demonstrated at 1 h when hepatic GSH was markedly depleted in response to APAP-induced chemical stress. This observation makes ophthalmic acid a potential biomarker of GSH consumption. However, at later time points, ophthalmic acid levels continue to rise despite recovering levels of hepatic GSH, possible explanations of which are discussed in chapter 4. For the purpose of our objectives in this current work, the less prominent rise in ophthalmic acid in the $Nrf2^{(-/-)}$ animals demonstrates the abrogated ability of these animals to up-regulate GCL activity for the synthesis of either ophthalmic acid or GSH.

Levels of methionine and taurine in the serum of $Nrf2^{(-/-)}$ control animals at 0 h were significantly lower compared with their wild type counterparts (Figure 3.5). This could be an indication of an adaptive mechanism employed by the $Nrf2^{(-/-)}$ animals to protect against cellular stress. As this difference was not seen in the liver, the decrease in the serum of these metabolites could be a method of conserving/maintaining hepatic levels which would be highly significant given the importance of both methionine and taurine in cell defence.

Although significant changes in metabolite levels were observed in both Nrf2 wild type and null animals in response to APAP treatment, the response is very similar between the two strains indicating that both employed similar cellular defence mechanisms in response to acute APAP treatment. The difference however is the level of response which is generally higher in the wild type compared with the Nrf2 nulls. This suggests that Nrf2 does play a role in the response of mice to acute APAP-induced stress in addition to other adaptive mechanisms.

3.4.2 Chronic study with escalating doses of (150-600 mg/kg) APAP

Work described in this section was carried out to determine: 1) if the mouse is able to adapt to chemical-induced stress; 2) if this adaptation is Nrf2-mediated; and, 3) if this adaptation involved modulations within the GSH system.

Escalating doses of APAP over an eight-day treatment regimen resulted in only a mild/no ALT response, despite the final dose of 600 mg/kg being invariably toxic when administered acutely. Although not determined to be statistically significant, the marked increase in serum ALT of the $Nrf2^{(-/-)}$ animals compared with the $Nrf2^{(+/+)}$ in response to chronic APAP treatment (Figure 3.6) is consistent with results presented in the acute study (Figures 3.3) as well as reports in the literature (Chan and Kwong, 2000, Chan *et al.*, 2001, Enomoto *et al.*, 2001) further demonstrating the enhanced susceptibility of the $Nrf2^{(-/-)}$ animals to chemical stress. In addition, the $Nrf2^{(+/+)}$ animals were able to adapt to this APAP dosing regimen as demonstrated by the lack of increase in serum ALT at the end of the treatment indicating no or minimal liver injury. As this was not observed in the equivalent $Nrf2^{(-/-)}$ animals, this effect is at least in part, Nrf2 mediated.

The subtle changes observed in the majority of metabolites monitored in the GSH system of both $Nrf2^{(+/+)}$ and $Nrf2^{(-/-)}$ animals could be indicative of a limited role of the GSH system with regards to chronic APAP treatment. However, it may also be these subtle changes that are key in the adaptation of the $Nrf2^{(+/+)}$ animals at least, to chronic APAP treatment. Although these differences in metabolite levels between APAP treated and control groups were also observed in the $Nrf2^{(-/-)}$ animals, the inability of the $Nrf2^{(-/-)}$ APAP-treated group to attenuate the increase in serum ALT suggests that other factors in addition to modulation in metabolites within the GSH system are required for attenuation/protection from APAP-induced liver injury.

A number of metabolites were significantly altered in response to APAP treatment, however these differences were not comparable between the $Nrf2^{(+/+)}$ and $Nrf2^{(-/-)}$ animals. Interestingly, the difference between $Nrf2^{(+/+)}$ and $Nrf2^{(-/-)}$ mice may explain part of the protective mechanism employed by the $Nrf2^{(+/+)}$ mice and why this was not observed in the $Nrf2^{(-/-)}$ mice.

The significant decrease in serum methionine in the APAP-treated $Nrf2^{(+/+)}$ mice, which was not observed in the equivalent Nrf2 nulls could be indicative of a conservation mechanism for hepatic methionine, through modulation in methionine transport, for example, employed by the $Nrf2^{(+/+)}$ animals. This could have a significant impact on the ability of these animals to adapt to high levels of chemical stress as a consequence of APAP treatment, considering the role of methionine as an essential source of methyl groups and sulphur atoms, both of which are essential in sustaining physiological maintenance and defence of the cell (Finkelstein, 1998, Varela-Moreiras, 2001, Martinez-Chantar *et al.*, 2002,

Mato *et al.*, 2002). With regards to the GSH system, methionine is an essential source of cysteine and 2-AB, precursors of GSH and ophthalmic acid syntheses, respectively (Finkelstein, 1998). Given this role, the un-altered levels of both GSH and ophthalmic acid in these animals may therefore be in part due to the maintained levels of methionine, which may have subsequently led to the consistent supply of cysteine and 2-AB. The significance of methionine conservation in the liver could also be explained by the fact that it is an essential amino acid, therefore the inability of the body to synthetically up-regulate methionine levels may partly explain the decrease in serum methionine. The absence of this observation in the equivalent $Nrf2^{(-/-)}$ animals suggests that this response is Nrf2-mediated and is consistent with the difference in serum ALT between the treated $Nrf2^{(+/+)}$ and $Nrf2^{(-/-)}$ animals.

However, serum methionine in the $Nrf2^{(-/-)}$ control group was significantly lower compared with levels from the equivalent wild type group consistent with that observed in the serum of the control groups of both strains at 0 h in the acute APAP study. The $Nrf2^{(-/-)}$ may have therefore also employed the same mechanism as the wild type to conserve hepatic methionine levels, but as described above, this mechanism of maintaining hepatic methionine may not have been up-regulated more readily compared with the $Nrf2^{(-/-)}$ animals. This response is therefore Nrf2-mediated. This may be an adaptive mechanism employed by the Nrf2 nulls as a consequence of Nrf2 deletion, as although it's purpose as a source for cysteine may not be of significant use for GSH synthesis as the enzymes involved in this reaction, GCL and GS are under Nrf2 transcriptional regulation, cysteine is an antioxidant in its own right and would therefore still be used for cellular defence.

The significant increase in hepatic taurine of the $Nrf2^{(-/-)}$ APAP-treated animals compared with the $Nrf2^{(-/-)}$ control and equivalent wild type APAP-treated groups could be a defence mechanism adopted by these animals in response to chronic APAP treatment, taurine being known to possess a number of cytoprotective properties including antioxidant activity (Gordon *et al.*, 1986, Chen, 1993, Wang *et al.*, 1996, Lourenco and Camilo, 2002) and modulation of calcium homeostasis (Huxtable, 1992, Wang *et al.*, 1996). In particular, treatment with a pharmacological dose of taurine, prior to, simultaneously or 1 h after an acute toxic dose of APAP were shown to significantly attenuate liver injury (Waters *et al.*, 2001). Furthermore, the inability of the $Nrf2^{(-/-)}$ animals to utilise cysteine for the up-regulation of GSH synthesis may have led to higher levels of cysteine oxidation, a process in which taurine is a product, therefore contributing to the high levels of hepatic taurine observed.

No other significant changes were observed in the metabolites monitored in this study. As mentioned at the beginning of this section, subtle changes and not necessarily only significant ones that contribute to potential adaptaion of these animals to chemical stress, however, in addition to modulations within the GSH system, other mechanisms could also be in play that may have allowed these animals to an APAP-induced environment as will be discussed in chapter 6.

3.4.3 Chronic study with escalating (150-600 mg/kg) doses of APAP and subsequently challenged with a lethal dose

Work discussed in this section was carried out to establish whether chronic APAP treatment, employed as a form of preconditioning, affords mice protection from a subsequent toxic dose of APAP. The role of Nrf2 in APAP autoprotection within the GSH system was also investigated by comparing the response of both $Nrf2^{(+/+)}$ and $Nrf2^{(-/-)}$ mice. For clarification, four treatment regimens were employed in this experiment as summarised in Table 3.4.

Saline pretreatment did not afford protection to either strain against the toxic dose of APAP as demonstrated by the significant increase in serum ALT in the challenged animals ($Nrf2^{(+/+)}$SA and $Nrf2^{(-/-)}$SA). Although to a lesser extent, this was also observed in the APAP pretreated groups of both strains that were also challenged with a toxic dose of APAP ($Nrf2^{(+/+)}$AA and $Nrf2^{(-/-)}$AA). This demonstrates that the significant increase in serum ALT observed in the saline pretreated groups ($Nrf2^{(+/+)}$SA and $Nrf2^{(-/-)}$SA) was to some extent attenuated in the equivalent APAP pretreated groups ($Nrf2^{(+/+)}$ AA and $Nrf2^{(-/-)}$AA) indicating autoprotection and consistency with reports in the literature (Tredger *et al.*, 1995, Shayiq *et al.*, 1999, Aleksunes *et al.*, 2008). As this attenuation in serum ALT increase was observed in both the $Nrf2^{(+/+)}$ and $Nrf2^{(-/-)}$ APAP pretreated animals, autoprotection from APAP-induced stress pretreatment is not Nrf2-mediated.

The consistently lower levels of serum ALT in the $Nrf2^{(-/-)}$SA and $Nrf2^{(-/-)}$AA groups compared with the equivalent $Nrf2^{(+/+)}$ animals do not an accurately represent the relative toxicity in the $Nrf2^{(-/-)}$ and $Nrf2^{(+/+)}$ animals, as the average serum ALT presented here were only from those that survived. Treatment with a toxic dose of APAP in these groups resulted in deaths (Figure 3.9-B) indicating a high mortality rate in the $Nrf2^{(-/-)}$ animals compared with the $Nrf2^{(+/+)}$. As such, although Nrf2 is not the sole determinant of

autoprotection, it is clearly an important factor in the defence system of a mouse to combat against APAP-induced toxicity.

The increase in serum ALT observed in both the SA and AA groups of both strains was supported by the significant decrease in hepatic GSH, indicating APAP-induced stress (Figure 3.10). However in contrast to the attenuation in serum ALT increase in the AA groups, the degree of GSH depletion was comparable between the SA and AA groups of both strains. Within the context of the GSH system, autoprotection may have therefore been in part afforded from modulations within the GSH system as discussed in section 3.4.2 during APAP pretreatment but not from an up-regulation of GSH synthesis in response to the toxic APAP dose. In particular, modulations in serum methionine and hepatic taurine in the $Nrf2^{(+/+)}$A as well as taurine and ophthalmic acid in the $Nrf2^{(-/-)}$A may have enhanced the defence mechanism of these animals therefore allowing them to respond to the APAP challenge better. Consistent with its role in the GSH redox cycle, the depletion in hepatic GSH also led to the significant depletion in GSSG in the AS and AA groups of $Nrf2^{(+/+)}$ and $Nrf2^{(-/-)}$ animals (Figure 3.10).

The increase in hepatic cysteine observed in both $Nrf2^{(+/+)}$ and $Nrf2^{(-/-)}$SA groups could be indicative of the demand for cysteine for use in cellular defence such as GSH synthesis, in response to APAP toxicity. However, this increase was not reflected in the corresponding hepatic GSH data being a precursor for GSH as GSH recovery was not observed. This may be due to several factors including the rate of GCL and GS activities, which may have been subjected to oxidation and/or covalent binding given the high level of APAP-mediated stress present, therefore reducing or even abrogating GCL and/or GS activities. However, ophthalmic acid levels indicate that both GCL and GS are still fully active in the SA groups of both strains. This provides further support for a potential switch mechanism in the substrate affinity of GCL from cysteine to 2-AB, hence the significant increase in hepatic and serum OA in these animals. As the intact activity in GCL and GS are evident for OA synthesis in both strains, the proposed switch in substrate affinity of GCL is not likely to be Nrf2-mediated. Furthermore, this also suggests that this switching mechanism is carried out by existing hepatic GCL particularly in the $Nrf2^{(-/-)}$animals. In the $Nrf2^{(+/+)}$, this switch may have been carried out by a combination of existing and newly synthesised GCL. This increase in hepatic and serum ophthalmic acid was also evident in the APAP pretreated and challenged groups (AA) of both strains indicating that irrespective of pretreatment, the extremely toxic conditions posed by the challenge dose at day 9 (1000 mg/kg) may have led to all groups employing the same defence pathway to combat and

therefore survive these toxic conditions. In such situations, it would therefore be difficult to differentiate between the $Nrf2^{(-/-)}$ and $Nrf2^{(+/+)}$ with regards to their response. Alternatively, cysteine in its own right is an antioxidant, as demonstrated by the use of N-acetylcysteine as an antidote to APAP self-poisoning in the clinic. Therefore, although the significant increase in hepatic cysteine in the SA group was not reflected in an increase in hepatic GSH, it may have been utilised as a direct antioxidant for the detoxification of reactive species in the cell.

The significant decrease in hepatic methionine in the SA and AA groups of both strains indicate the high demand of methyl groups and sulphur atoms by various metabolic pathways including those in the GSH system during chemical stress. This, however was only reflected in the serum of the SA group of the $Nrf2^{(-/-)}$ animals (Figure 3.11) whilst levels in other groups were comparable with their respective controls. This difference in methionine levels in the $Nrf2^{(-/-)}$SA group between the liver and serum may be indicative of the demand for methionine in extrahepatic tissues, hence the decrease in serum, whilst the unaltered levels in the liver may be indicative of the abrogated ability of the $Nrf2^{(-/-)}$ animals to up-regulate GSH synthesis. As such, there is less demand for methionine in the liver.

Taurine levels in the liver were unaltered in all groups of both $Nrf2^{(+/+)}$ and $Nrf2^{(-/-)}$ mice. However consistent decreases in the serum SA and AA groups of both $Nrf2^{(+/+)}$ and $Nrf2^{(-/-)}$ mice indicate a response to APAP treatment. As taurine has been reported to possess cytoprotective properties, its decrease in serum may reflect the demand by various tissues including the liver to combat against APAP toxicity. Absorption of taurine from the circulation therefore supports the unaltered levels observed in the liver of all groups. However, taurine may also be formed through cysteine oxidation and therefore the maintenance of hepatic taurine may be a combination of both mechanisms.

3.4.4 Overall conclusion

Under the three dosing regimens employed in this chapter, the $Nrf2^{(-/-)}$ animals were demonstrated to be more susceptible to APAP-induced stress compared with the $Nrf2^{(+/+)}$. This difference was more prominent in the serum ALT data rather than in the selected metabolites measured in the GSH system. This suggests that perturbations within the GSH system do not indicate toxicity or demonstrate the effect of Nrf2 deletion in the $Nrf2^{(-/-)}$ animals, as observed throughout the three studies. Instead, what the studies have shown is

that the response of both strains to the three APAP dosing regimens were very similar. As discussed in section 3.4.1, both strains responded similarly up to a certain threshold of chemical stress. When levels of stress goes beyond this threshold and therefore requires up-regulation of Nrf2-mediated defence pathways, differences between the strains are then observed. However, monitoring metabolites in the GSH pathway does not demonstrate significant effects, if any, of deleting the Nrf2 gene. As such, other pathways are worth investigating to further characterise the effect of Nrf2 deletion in mice.

Chapter 4

Qualification of ophthalmic acid as a potential biomarker of chemical stress *in vitro* and *in vivo*

TABLE OF CONTENTS

4.1 INTRODUCTION

Current research and clinical practice rely on the use of biomarkers to differentiate the pathology of the diseased state versus normal, as well as to characterise the response of the body to therapeutic interventions. In particular, research in DILI uses a plethora of biomarkers to indicate the window of efficacy to toxicity caused by drugs (Ozer *et al.*, 2008). However, despite the significant role in the study of DILI, the discovery of a biomarker that fulfils all the desired attributes described in chapter 1 has to this day proven a challenge. In response to this, many groups continue to expend considerable effort into the discovery and development of new (and existing) biomarkers to maintain a standard of high quality in research, including that of DILI, with the ultimate aim of benefitting public health.

Alanine amino transaminase (ALT) is one such biomarker that has been extensively used as a marker of liver injury (Amacher, 1998, Amacher, 2002). Its role as a marker for DILI has been attributed to its high hepatic expression (Sherman, 1991, Dufour *et al.*, 2000b, Dufour *et al.*, 2000a) compared with its low abundance in extrahepatic tissues both in rodents and human (Boyd, 1983). As such, significant elevations in serum ALT activity have been correlated with its leakage from the liver into the circulation during liver injury. Furthermore, its sensitivity to DILI where its levels are highly elevated in serum makes it an amenable biomarker in practise. However, its specificity is dampened by its distribution in extraheptic tissues and elevation in serum even in the absence of DILI (Edgar *et al.*, 1998, Thulin *et al.*, 2008).

Glutathione is another marker that has been invaluable in the study of DILI. It has an extensive list of functions both in basic cellular processes (Meister, 1983, Wu *et al.*, 2004, Townsend, 2007) and in cell defence (Meister, 1988, DeLeve and Kaplowitz, 1991, Fernandez-Checa *et al.*, 1992) under physiological conditions and during cellular stress. This, along with its high abundance in the liver, demonstrates its relevance and therefore usefulness as a marker of oxidative stress. Indeed, a number of well known hepatotoxicants including carbon tetrachloride (CCl_4) (Recknagel *et al.*, 1989), APAP (Hinson *et al.*, 1981), bromobenzene (Jollow *et al.*, 1974) and furosemide (Williams *et al.*, 2007) are also known to perturb the hepatic GSH store. However, despite all these, its utility is limited to preclinical research as a consequence of its chemical instability as detailed in the chapter 1. As such, a stable and accessible biomarker that directly indicates perturbations in hepatic

GSH could prove highly valuable in the study of DILI, particularly in the clinic where the measurement of hepatic GSH is rarely, if ever, an option.

In a recent metabolomics study that looked at the hepatic metabolic profile of mice treated with APAP, ophthalmic acid, an analogue of GSH, was observed to increase significantly in the liver and serum as GSH was depleted. As such, ophthalmic acid was proposed to be a direct biomarker of GSH consumption (Soga *et al.*, 2006). Furthermore, the same group also demonstrated similar results in mice treated with two other modulators of GSH, diethyl maleate (DEM), which directly binds to the thiol group of GSH (Zalups and Lash, 1997) and buthionine sulfoximine (BSO), which inhibits the enzyme responsible for the rate-limiting step of GSH synthesis (Griffith and Meister, 1979). Ophthalmic acid utilises the same synthetic enzymes responsible for GSH biosynthesis, glutamate cysteine ligase (GCL) and glutathione synthetase (GS), however, GSH and ophthalmic acid are chemically different in that the thiol-containing precursor, cysteine, required for GSH synthesis is replaced by 2-aminobutyrate (2-AB) during ophthalmic acid synthesis, 2-AB being an amino acid that shares a similar structure to cysteine but with the thiol group replaced with a methyl group instead (Figure 1.8). Based on these experiments, Soga *et al* (2006) suggested that the elevation in ophthalmic acid both in the liver and serum of APAP-treated mice may be due to a combination of cysteine/GSH depletion and subsequent activation of glutamate cysteine ligase (GCL), the enzyme responsible for the rate-limiting step of GSH synthesis, towards the ophthalmic acid biosynthetic route.

Whilst no research has been done to specifically elucidate the function of ophthalmic acid, it has been extensively used to investigate the significance of the thiol group of GSH in its various functions, with most studies predominantly focussed on GSH-mediated transport (Ballatori and Dutczak, 1994, Leslie *et al.*, 2001, Leslie *et al.*, 2003, Leslie *et al.*, 2004, Circu *et al.*, 2009). As such, work described in this chapter was aimed at evaluating the extent to which ophthalmic acid qualifies as a surrogate for GSH consumption and consequently represents a potential biomarker of oxidative stress. This was investigated in both *in vivo* and *in vitro* settings using mouse, rat and human model systems as well as a variety of GSH modulating agents including APAP, its reactive metabolite NAPQI and DEM. Ophthalmic acid and GSH were measured in order to elucidate the relationship between these two metabolites during cellular stress. ALT and mitochondrial activities were also measured to indicate toxicity where appropriate. Experiments described in this chapter were therefore carried out to address the following: 1) Is ophthalmic acid a direct biomarker of GSH consumption or a general biomarker of

oxidative stress? and 2) Can ophthalmic acid be used as a biomarker of oxidative stress across species in a variety of *in vitro* and *in vivo* model systems?

4.2 MATERIALS AND METHODS

4.2.1 Materials

All chemicals and reagents were purchased from Sigma-Aldrich (Poole, Dorset, UK) unless otherwise stated. Ophthalmic acid (OA) was purchased from Bachem (Weil am Rhein, Germany). Solvents were obtained from either VWR (Lutterworth, Leicestershire UK) or Fischer Scientific (Loughborough, Leicestershire, UK). THLE cells and chemicals used in sections 4.2.7 and 4.2.8 are detailed in chapter 2 section 2.2. Hepa1c1c7 was purchased from ATCC-LGC Standards (Teddington, Middlesex, UK). Infinity ALT (GPT) liquid stable reagent was purchased from Thermo Scientific (Loughborough, Leicestershire, UK).

4.2.2 Animals

Adult male Wistar rats and adult male C57BL/6 mice were obtained from Charles River Laboratories (Margate, Kent, UK). All animal experiments were undertaken in accordance with criteria outlined in a license granted under the Animals (Scientific Procedures) Act 1986, and approved by the Animal Ethics Committee of the University of Liverpool. All animals were housed under 12-h light/dark cycles with room temperature maintained between 19 – 23 °C. Animals had free access to food and water.

4.2.3 APAP treatment of mice and its effect on ophthalmic acid levels

Male C57BL/6 mice (20 – 25 g) were administered with 530 mg/kg APAP in 0.9 % saline or vehicle (control) i.p. for 0, 1, 2, 4, 6 and 24 h and sacrificed at each time point by CO_2 asphyxiation. Livers were removed and snap frozen in liquid nitrogen. Serum samples were prepared by allowing blood samples collected to clot overnight at 2 – 4 °C followed by pulse centrifugation at 4000 rpm. All samples were stored at -80 °C until analysis.

4.2.4 DEM treatment of rats and its effect on ophthalmic acid levels

Male Wistar rats (200 – 300 g) were anaesthetised (1.4 g/ml urethane in saline dosed at 1.0 ml/kg i.p) and cannulated via the trachea and carotid artery fed with heparin containing saline (250 U/ml). A 3 cm incision in the abdomen was also made, and covered with saline soaked gauze to allow access and collection of liver sections during sampling. Blood (including pre-dose) and liver samples were collected simultaneously at 15 minute intervals up to and including the 2 h time point. Blood samples which contained heparin were centrifuged at 10, 000 rpm for 5 min to allow the isolation of serum. Liver and serum samples were immediately snap frozen in liquid nitrogen and stored at -80 °C until analysis.

4.2.5 Culture of Hepa1c1c7 cells

The mouse hepatoma cell line Hepa-1c1c7 was maintained in Dulbecco's modified Eagle's medium (cDMEM) supplemented with L-glutamine (584 mg/L), foetal bovine serum (FBS; 10%), penicillin (100 U/mL) and streptomycin (100 µg/mL) in a humidified chamber at 37 °C under 5 % CO_2. For drug preparation and incubations, un-supplemented media (DMEM) was used.

Cells were grown in a T75 Nunclon Δ culture flask (75 cm^2) and routinely passaged when growth reached 80 % confluency. Briefly, cDMEM was removed and the cells were washed with DMEM to remove the majority of protein from the growth media. The cells were detached from the surface of the culture flask by the addition of 3 mL trypsin and incubation for 5 min at 37 °C. The detached cells were resuspended in 10 mL cDMEM and pelleted by centrifugation at 1000 rpm for 5 min at room temperature. The trypsin/cDMEM supernatant was carefully removed and the cells were resuspended in fresh 10 mL cDMEM. Cell clumps were broken up by passing the cell suspension through a 21-gauge needle using a 5 mL syringe. For future experiments, the cells were re-seeded at a ratio of 1:4 (cells:cDMEM). Cells used were between 1 – 12 passages.

Accurate seeding of cells for a particular experiment was achieved by making a mixture of cell suspension (40 µl) as described above and Trypan Blue (0.4 % w/v; 10 µl) and counting 10 µl of this mixture using a haemocytometer and a light microscope (Wilovert D6330) at 20X magnification. Viable cells (Trypan Blue positive) within the central 5 x 5 square (0.1 mm^3) were counted and calculated as follows:

Number of cells x 1.1 (to correct for dilution of cell suspension) (40 µl) with Trypan Blue (10 µl) = cells per 0.1 mm^3 x 10,000 = cells/1 cm^3 = cells/1 mL.

4.2.6 Treatment of Hepa1c1c7 cells with NAPQI and its effect on ophthalmic acid levels

Hepa1c cells were seeded (250 x 10^3 cells/well) onto Nunclon Δ 6-well plates (culture area of 9.6 cm^2/well) in a total volume of 2 mL cDMEM and left to grow for 24 h. Prior to drug treatment, the growth media was removed and the cells were washed with HBSS to remove the remaining protein from the growth media. The cells were subsequently treated with increasing concentrations (0 – 300 µM in 0.1 % DMSO) of NAPQI and incubated for 1 or 24 h in a humidified chamber at 37 °C under 5 % CO_2. After incubation, the treatment media was removed and the cells were washed with HBSS. The cells were lysed by four freeze-thaw cycles in 300 µl 0.1 % formic acid, collected in eppendorf tubes and stored at -80 °C until analysis.

4.2.7 DEM treatment of THLE-2E1 and THLE-Null cells and its effect on ophthalmic acid level

THLE-Null and THLE-2E1 cells were seeded (450 x 10^3 cells/well) in a Biocoat™ 6-well plate for 24 h in complete Basal PMFR-4 media (CM; prepared as detailed in chapter 2, section 2.2). Each cell line was treated with increasing concentrations of DEM (0 – 1000 µM prepared in serum free Basal PMFR-4 media (SFM) prepared as detailed in chapter 2, section 2.2 in triplicate and left to incubate for 1 h in a humidified chamber at 37 °C under 5 % CO_2. Treatment media was discarded and the cells were washed with HBSS (-Ca^{2+}/-Mg^{2+}). To each well, 0.1 % formic acid (250 µl) was added and subsequently subjected to four freeze-thaw cycles. Samples were collected in eppendorf tubes and stored at -80 °C until analysis.

4.2.8 APAP treatment of THLE-2E1 and THLE-Null cells and its effect on ophthalmic acid levels

Cell lysates obtained from chapter 2, section 2.2 for both THLE-Null and THLE-2E1 were also analysed for ophthalmic acid levels as detailed in section 4.2.11.

4.2.9 Determination of protein content

Protein content of liver homogenates prepared in sections 4.2.3 and 4.2.4 were measured following the method of Lowry *et al.* (1951) as detailed in chapter 2 section 2.2. Protein content in both THLE and Hepa1c1c7 cell lysates treated with DEM or NAPQI were measured following the method of Bradford (1976) as detailed in chapter 2 section 2.2 . Treatment of the THLE cells with APAP resulted in very low protein content at higher APAP concentrations and longer incubation periods, therefore protein content was measured following the protocol of Lowry *et al.* (1951) as detailed in chapter 2 section 2.2.

4.2.10 Determination of GSH content

Hepatic GSH from the *in vivo* studies were determined following the method of Vandeputte *et al.* (1994) as detailed in chapter 3 section 3.2.

4.2.11 Determination of ophthalmic acid by LC-MS-MS

Serum and liver homogenate samples from the *in vivo* studies were obtained, prepared and analysed as detailed in chapter 3 section 3.2. Cytosolic extracts from the *in vitro* studies were analysed using the same LC-MS-MS conditions employed in chapter 3.

4.2.12 Statistical analysis

Where applicable, all data were expressed as mean ± standard error of the mean (SEM). Values to be compared were analysed for non-normality using a Shapiro-Wilk test. Unpaired t-tests or one-way ANOVA was used when normality was indicated. A Mann-Whitney U or Kruskall-Wallis test was used for non-parametric data. All calculations were

performed using StatsDirect statistical software and results were considered to be significant when $p < 0.05$.

4.3 RESULTS

4.3.1 Correlation between GSH and ophthalmic acid in APAP-treated mice

Glutathione and ophthalmic acid levels in the liver and serum of C57BL/6 mice administered a toxic dose of APAP (530 mg/kg) were measured by LC-MS-MS to validate the proposed potential role of ophthalmic acid as an indicator of GSH consumption. ALT activity was also measured as an independent marker of toxicity.

Significant hepatic GSH depletion was observed in the APAP-treated animals compared with their respective time-matched controls up to and including the 4 h group. A similar trend was also observed with the 6 h group although the difference was not determined to be statistically significant. In contrast, comparable levels of hepatic GSH were observed between the treated and control animals in the 24 h group (Figure 4.1 C).

The significant difference in the levels of serum ALT at 6 and 24 h in the APAP-treated animals compared with their respective time-matched controls indicated APAP-induced toxicity (Figure 4.1 D). Furthermore, this demonstrated serum ALT as a late marker of liver toxicity.

In contrast to the significant depletion observed in hepatic GSH, hepatic ophthalmic acid was significantly higher in the APAP-treated animals compared with control in the 1, 2 and 6 h groups whilst comparable levels were observed in the 0 and 24 h groups. Interestingly, the trend observed in hepatic ophthalmic acid was not directly reflected in the serum as only the APAP-treated animals in the 4 and 6 h groups presented significantly higher levels of ophthalmic acid in serum compared with their respective time-matched controls (Figure 4.1 A and B).

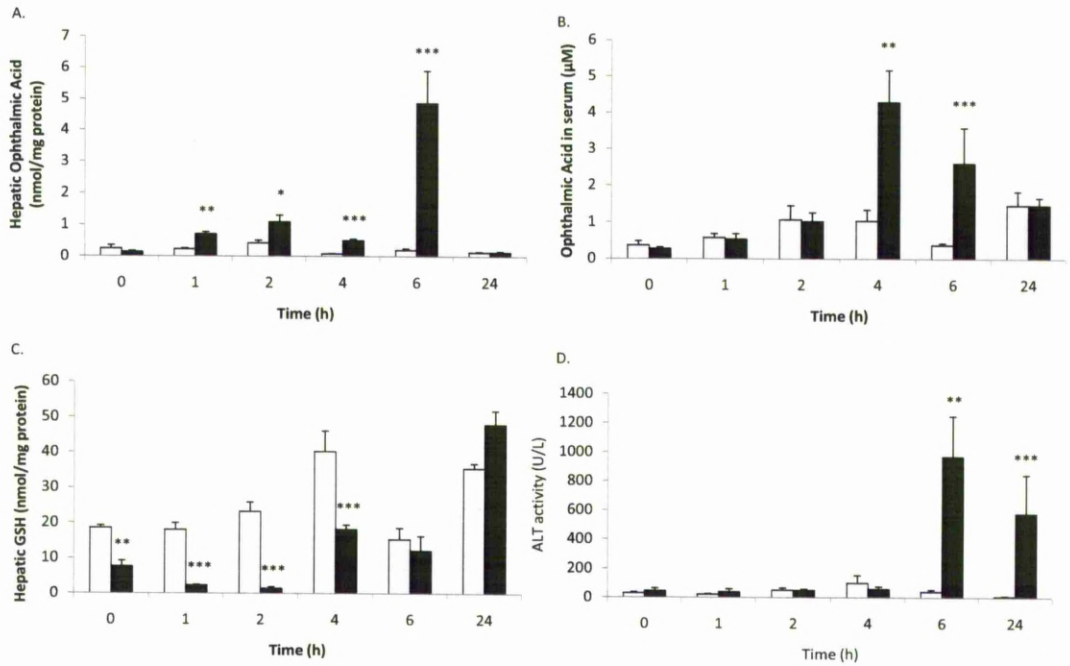


Figure 4.1 The effect of APAP-mediated chemical stress in mouse liver and serum. C57BL/6 male mice were administered 530 mg/kg APAP in 0.9 % saline (■) or an equivalent amount of vehicle as control (□) i.p. for 0, 1, 2, 4, 6 and 24 h. Animals from each group were sacrificed at the end of their designated treatment periods by CO_2 asphyxiation. Ophthalmic acid levels were measured both in the liver (A) and serum (B) samples. Hepatic GSH (C) and serum ALT activity (D) were also measured to identify APAP-induced oxidative stress and toxicity respectively. * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$ against time-matched control. Each column represents the mean of four animals (n=4) and the bars indicate the standard error of mean (SEM).

4.3.2. Correlation between GSH and ophthalmic acid in DEM-treated rats

To determine whether the inverse correlation between GSH and ophthalmic acid in the APAP-treated mice (Figure 4.1) also applies to other chemical modulators of GSH in other species, male Wistar rats were treated with 150 mg/kg DEM and measured for both GSH and ophthalmic acid in the liver and blood samples collected at pre-dose and 15 minute intervals post-dose up to and including the 2 h time-point.

As demonstrated in Figure 4.2, hepatic GSH was significantly depleted at all time points measured compared with the pre-dose value. Although not determined to be significant, an increasing trend in ophthalmic acid both in the liver and serum of each animal was observed, with increases in ophthalmic acid seen to be higher in the serum than in the liver.

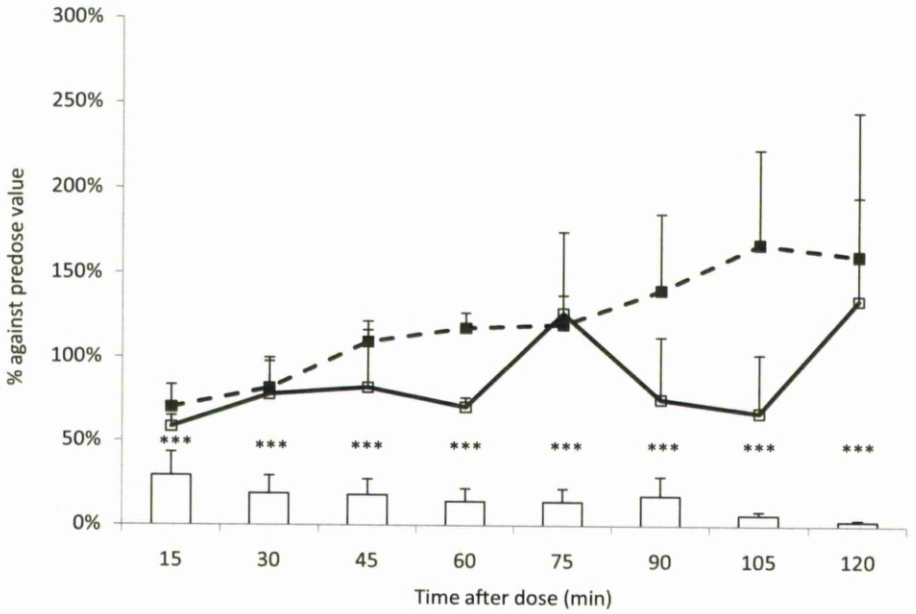


Figure 4.2 Ophthalmic acid and GSH levels DEM-treated rats. Male Wistar rats were administered 150 mg/kg DEM. Blood and liver snips were collected simultaneously at pre-dose and at 15 minutes intervals from 0 minute post-dose up to and including 120 minutes. Ophthalmic acid levels in both liver (—▲—) and serum (—■—) as well as hepatic GSH (□) were measured. Data presented are percentage of the corresponding pre-dose value. Error bars represent the standard error of mean (SEM), n = 4. *** p≤ 0.001 against the pre-dose group.

4.3.3 Correlation between GSH and ophthalmic acid in Hepa1c1c7 mouse cells treated with DEM

Hepa1c1c7 hepatocarcinoma cells were treated with increasing concentrations (0 – 1000 µM) of DEM and were analysed for both GSH and ophthalmic acid levels to determine whether observations made *in vivo* as demonstrated in sections 4.3.1 and 4.3.2 could be modelled *in vitro*. A concentration-dependent decrease in GSH compared with control was observed, with significantly lower levels in cells treated with DEM at concentrations between 250 to 1000 µM. On the other hand, no significant change was observed in ophthalmic acid in the same DEM-treated cells compared with control (Figure 4.3).

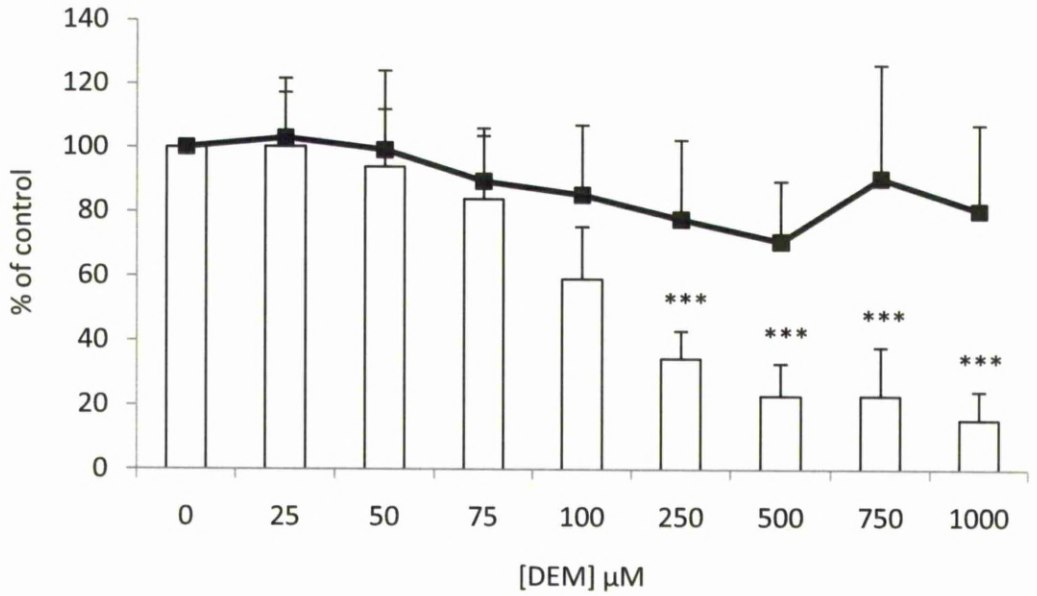


Figure 4.3 Glutathione and ophthalmic acid levels in mouse Hepa1c1c7 cells treated with DEM. Hepa1c1c7 cells were treated with increasing concentrations of DEM (0 – 1000 μM) in triplicate for 1 h. Cells were harvested and analysed for GSH (□) and ophthalmic acid (▬). Data presented are percentage of pre-treatment levels (0 h). Error bars represent the standard error of mean (SEM) of three independent experiments (n = 3). *** $p \leq 0.001$ compared with pre-treatment (0 h).

4.3.4 Correlation between GSH and ophthalmic acid in Hepa1c1c7 mouse cells treated with NAPQI

Hepa1c1c7 mouse cells were treated with the APAP reactive metabolite, NAPQI, to determine whether the correlation made between GSH and ophthalmic acid in the APAP-treated mice in section 4.3.1 could be modelled *in vitro*.

GSH levels measured (Figure 4.4) in cells with 300 μM NAPQI for 1 h demonstrated significant GSH depletion compared with control. Ophthalmic acid levels in these cells however did not demonstrate a significant change. In contrast, no significant difference in GSH levels was observed between control cells and those treated with NAPQI for 24 h. However, ophthalmic acid was significantly higher at 300 μM compared with control. This may be indicative of a potential alternative mechanism of ophthalmic acid increase in addition to or instead of GSH depletion.

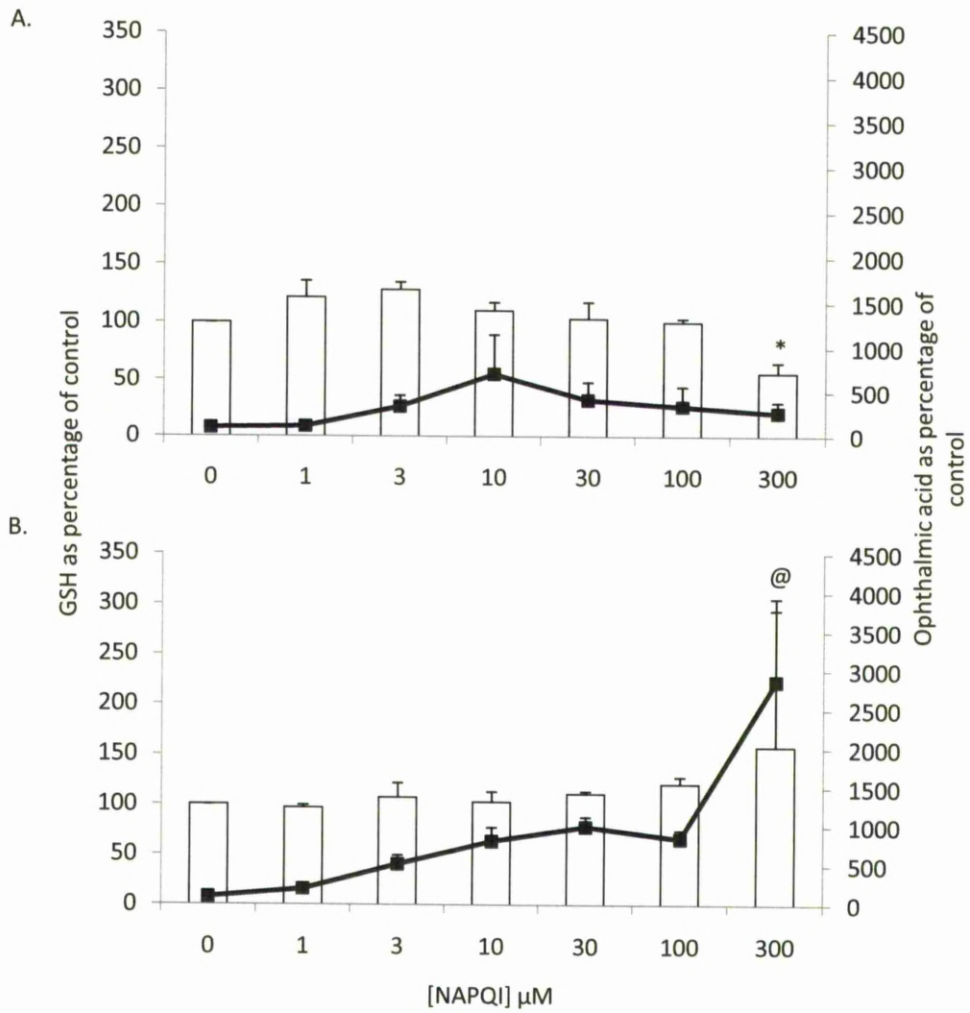


Figure 4.4 Glutathione and ophthalmic acid levels in Hep1c1c7 cells treated with NAPQI. Hepa1c1c7 cells were treated with increasing concentrations of NAPQI (0 – 300 μM) for 1 (A) or 24 (B) h in triplicate. After treatment, cells were harvested and analysed for GSH (□) and ophthalmic acid (▬) levels. Data presented are percentage of control (0 h). Error bars represent the standard error of mean of three independent experiments (n =3). * and @ represent $p \leq 0.05$ for GSH and ophthalmic acid respectively against corresponding controls.

4.3.5 Correlation between GSH and ophthalmic acid in THLE human liver cells treated with DEM

Ophthalmic acid levels were also investigated in the human liver epithelial cell lines, THLE-Null and THLE-2E1 to evaluate the transferability of ophthalmic acid as a biomarker across species in pre-clinical studies as an early indicator of potential toxicity in drug discovery. Treatment with DEM demonstrated significant GSH depletion in both cell lines (Figure 4.5).

Interestingly, this depletion in GSH was complemented by a trend towards increasing ophthalmic acid levels at increasing concentrations of DEM in the THLE-Null cell line (Figure 4.5 A). This however was not observed in the THLE-2E1 cells as no significant change was observed in ophthalmic acid despite the significant decrease in GSH at 500 and 1000 μM DEM (Figure 4.5 B).

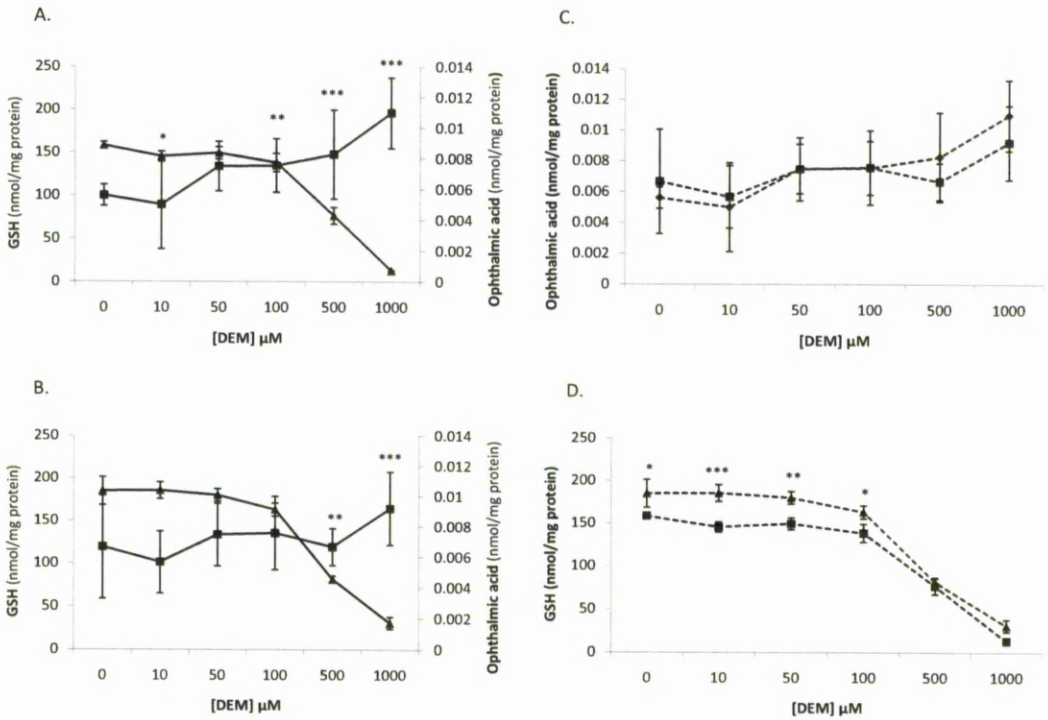


Figure 4.5 Glutathione and ophthalmic acid levels in THLE-Null and THLE-CYP2E1 cells treated with DEM. THLE-Null and THLE-CYP2E1 cells were treated with increasing concentrations of DEM (0 – 1000 μM) for 1 h in triplicate. After treatment, cells were harvested and analysed for GSH and ophthalmic acid levels. **A** represents GSH (—▲—) and ophthalmic acid (—■—) levels in the THLE-Null cells whilst **B** represents both metabolites in the THLE-2E1 cells. C and D are comparisons of ophthalmic acid and GSH respectively in the THLE-Null (—■-) and THLE-2E1 (—▲-) cells. Data presented are percentage of control (0 μM). Error bars represent the standard error of mean of three independent experiments (n = 3). * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$.

4.3.6 Correlation between GSH and ophthalmic acid in THLE cells treated with APAP

THLE-Null and THLE-2E1 cells were treated with increasing doses of APAP (0 – 25 mM) for 24, 48 and 72 h and were analysed for GSH and ophthalmic acid levels.

APAP treatment of THLE-Null and THLE-2E1 cells resulted in a concentration-dependent decrease in GSH with marked GSH depletion observed in the THLE-2E1 at all three time points but was only apparent in the THLE-Null cells at 48 and 72 h (Figure 4.6).

No trend was observed in ophthalmic acid levels both in the THLE-Null and THLE-2E1 cells at 24 h (Figure 4.6). However, ophthalmic acid in THLE-Null cells treated with 25 mM APAP for 48 and 72 h were markedly decreased which is in contrast with that observed in the THLE-2E1 where levels were increased. This contrast in observation may be indicative of the role of CYP2E1 in the toxicity and GSH depletion observed in the THLE-2E1 cells as presented in chapter 2 which would not be the reason for the toxicity and GSH depletion observed in the THLE-Null cells.

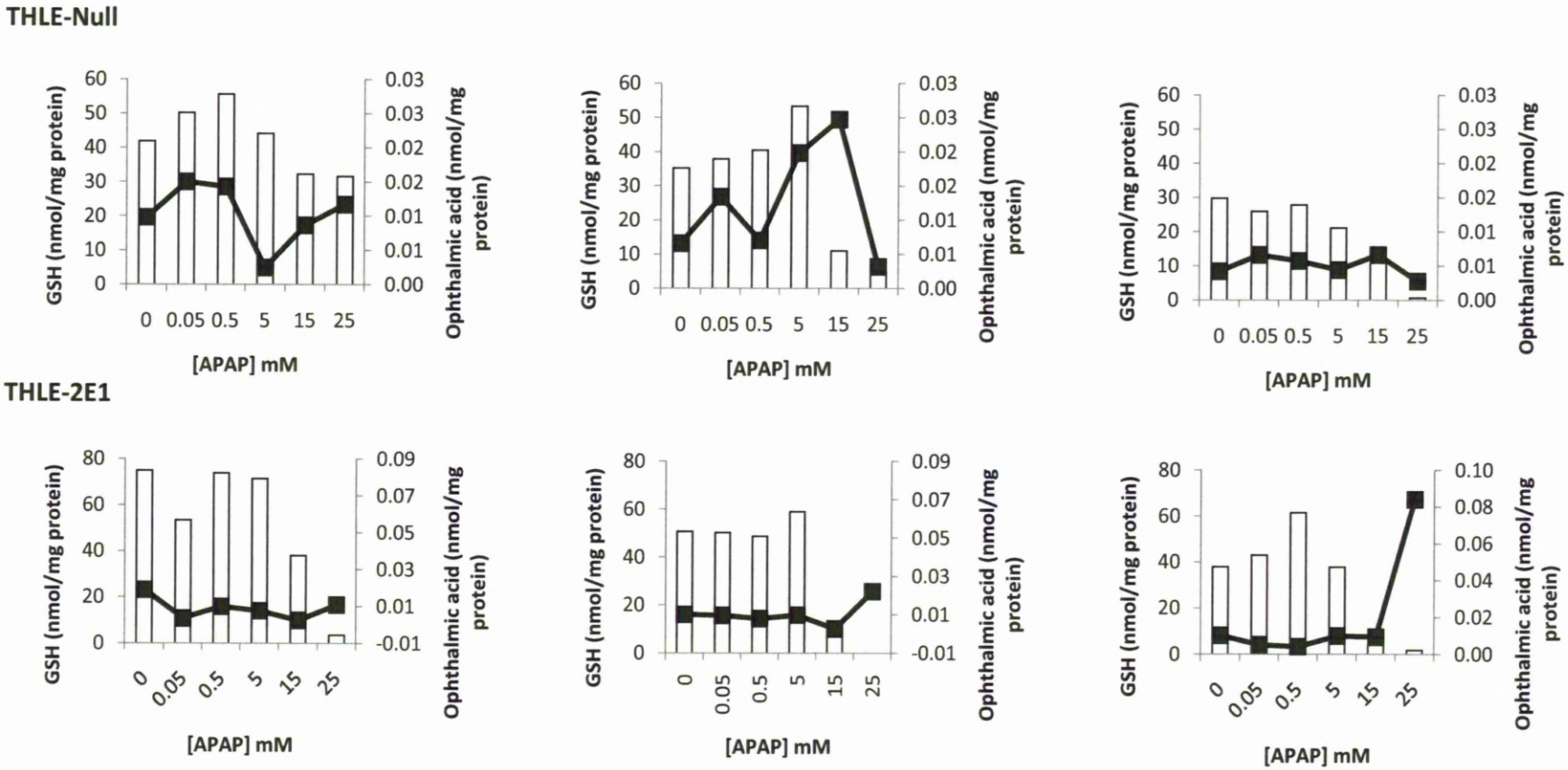


Figure 4.6 Glutathione and ophthalmic acid levels in THLE-Null and THLE-2E1 cells treated with APAP. THLE-Null and THLE-CYP2E1 cells were treated with increasing doses of APAP for 24 (A), 48 (B) or 72 (C) h. After treatment, cells were harvested and analysed for GSH (□) and ophthalmic acid (▬) levels. Data presented are percentage of control (0 mM) from two independent experiments.

4.4 DISCUSSION

The discovery and development of biomarkers is essential for the advancement of drug safety science. With respect to DILI, the usefulness of a biomarker that reflects GSH consumption is evident through its role not only in cell defence (Hayes and Pulford, 1995, Reed, 2004), but also in other fundamental cellular processes such as protein synthesis and cell proliferation (Meister, 1983, Wu *et al.*, 2004, Townsend, 2007). As such, and based on recent findings, this chapter focussed on the qualification of ophthalmic acid as a potential biomarker of GSH consumption (Soga *et al.*, 2006). Initial work in mice administered a toxic dose of APAP was carried out to verify findings by another group of the potential role of ophthalmic acid as a biomarker of GSH consumption (Soga *et al.*, 2006). Upon confirmation of this, ophthalmic acid was also measured in the rat treated with another GSH depleting agent, DEM, to elucidate whether ophthalmic acid can be used as a biomarker across a variety of experimental models *in vivo* and a range of GSH modulators. This was followed by work in the mouse hepatoma cell line, Hepa1c1c and human epithelial liver cell lines THLE-Null and THLE-2E1, using APAP or its reactive metabolite, NAPQI, as the GSH modulating agents to determine if ophthalmic acid can be employed as a biomarker of oxidative stress *in vitro*.

Administration of an acute toxic dose of APAP in mice resulted in the immediate depletion of hepatic GSH (Figure 4.1-C). In parallel to this but not a direct reflection of hepatic GSH depletion was the significant increase in hepatic ophthalmic acid (Figure 4.1-A). Furthermore, and of paramount interest with respect to biomarker development, this increase in hepatic ophthalmic acid was also observed in the serum (Figure 4.1-B). These initial results are in broad agreement with findings reported by Soga and colleagues (2006) and confirm the potential of ophthalmic acid as a biomarker of GSH consumption. However, upon closer inspection of our results (Figure 4.1), whilst hepatic GSH depletion occurred almost immediately, the increase in ophthalmic acid both in the liver and serum were more prominent at the later time points, demonstrating a delay in ophthalmic acid response to GSH depletion. As such, although there is a genuine correlation between GSH depletion and ophthalmic acid increase during xenobiotic-induced stress, as demonstrated in our lab, ophthalmic acid is not a direct marker of hepatic GSH consumption. Despite this, the ophthalmic acid response that has been consistently observed in this chapter and in the work described in chapter 3, and in other laboratories (Soga *et al.*, 2006) in response to xenobiotic-induced stress suggests that ophthalmic acid may still prove useful, as a general

biomarker of xenobiotic-induced stress either individually or when used as part of a panel. Further work to investigate the mechanism of this response observed in ophthalmic acid could be valuable in determining its specificity as a marker of oxidative stress.

What seemed like a delayed response in ophthalmic acid in our study compared with that reported in the literature may be accounted for by certain differences in the experimental protocols employed. In particular, whilst animals used in our study were not subjected to dietary restrictions, animals used by Soga and colleagues (2006) were starved overnight. Considering the similarities in the synthesis of ophthalmic acid and GSH (Figure 1.8) with regards to precursors and enzymes, diet may have been an important factor in the levels of GSH and ophthalmic acid. Indeed, in rats that were fed an 8 % casein diet supplemented with 3 % methionine (a precursor of cysteine and 2-aminobutyrate (2-AB)), GSH and 2-AB levels were observed to have increased whilst ophthalmic acid levels decreased (Takanori Kasai and KIRIYAMA, 1989a). This study also demonstrated that GCL has a higher affinity for the substrate cysteine compared with 2-AB, both of which are products of methionine metabolism, with Km values reported to be 0.3 and 1.0 mM for cysteine and 2-AB respectively (Seelig and Meister, 1985). As such, due to the consistent availability of cysteine in our animals, GCL activity would have been primarily involved in the synthesis of GSH. However, when cysteine supply is exhausted, not just for GSH synthesis but also for its antioxidant activity during xenobiotic-induced oxidative stress such as with APAP, GCL activity may have diverted to the synthesis of ophthalmic acid where 2-AB supply is plentiful, hence the later maximal peak of this metabolite. On the other hand, fasting of animals overnight prior to APAP administration (Soga *et al.*, 2006) may have provided enough time for GCL activity to adapt to such conditions which may consist/involve low methionine levels and may have consequently directed GCL and GS activities for ophthalmic acid synthesis as 2-AB is also a product of other metabolic pathways such as in the threonine metabolic pathway. Indeed, it has been reported that in starved animals, although threonine dehydrogenase, the dominant enzyme responsible for the metabolism of threonine to 2-ketobutyrate (another precursor of 2-AB) is inhibited, an alternative and less common pathway catalysed by threonine dehydratase is up-regulated accounting for a similar level of total hepatic threonine catabolic activity (77 – 86 %) to that of threonine dehydrogenase (87 %) (Bird and Nunn, 1983). The maintained supply of 2-AB may have therefore, in part, accounted for the earlier maximal peak of ophthalmic acid previously reported in starved animals (Soga *et al.*, 2006).

Administration of DEM to rats resulted in a significant time-dependent decrease in hepatic GSH with a concurrent, but non-significant increase, in ophthalmic acid both in the liver and serum (Figure 4.2). These findings are in support of the results in the mouse *in vivo* work with APAP (Figure 4.1) as it also demonstrates that ophthalmic acid is not a direct marker of GSH consumption. However, the trend towards increasing levels of ophthalmic acid as GSH is depleted also provides further support for the role of ophthalmic acid during oxidative stress. As this increase in ophthalmic acid was not determined to be significant, this suggests that other factors may be involved that influence this increase. Possible determining factors could be a certain threshold of GSH/ROS levels or the effect of oxidative stress resulting from GSH consumption on metabolic pathways that influence the synthesis of ophthalmic acid. It is also possible that GSH itself is not the determining factor in the GSH synthetic pathway that results in ophthalmic acid increase. Instead, it could be levels of the precursors involved. In particular, cysteine could be a potential candidate as it is the likely molecule to be affected during chemical stress. Its depletion may therefore be a more crucial factor for ophthalmic acid synthesis.

The increase in ALT in the APAP-treated mice, which is in practice used as a marker of toxicity in research and in the clinic, may have a role in the rise in ophthalmic acid increase as its catalytic activity results in the production of glutamate. Due to the nature of the study in rats with DEM, ALT could not be analysed as blood was collected every 15 minutes for 2 h from each animal. Sample volume was limited, allowing only the analysis of ophthalmic acid. It would be interesting to see if the correlation made between ALT and ophthalmic acid in mice treated with APAP can also be seen in the rat treated with DEM as this could provide further insight into the mechanism of ophthalmic acid increase, which is clearly not entirely if at all GSH-mediated. To investigate the correlation, if any, between ALT and ophthalmic acid, preliminary work would have to be carried out to determine when ALT levels are increased under this treatment regimen in rats with DEM. This will therefore aid in determining when to measure ophthalmic acid, GSH and ALT and make all the appropriate correlations.

In vitro studies carried out in the liver cell lines mouse Hepa1c1c7 (Figure 4.3) and human THLE-Null and THLE-2E1 (Figure 4.5) with DEM, were in agreement with the *in vivo* rat study, as a dose-dependent decrease in GSH with no significant change in ophthalmic acid levels were also observed. This further supports our previous suggestion of a multi-factorial mechanism of ophthalmic acid increase.

The trend towards increasing ophthalmic acid in the THLE-Null cells, in response to DEM treatment, which was not observed in the THLE-2E1 cells may be due to the difference in CYP2E1 expression between these two cell lines. As discussed in chapter 2, CYP2E1 overexpression leads to an increase in ROS production due to its loose coupling with the enzyme CPR (Gorsky *et al.*, 1984, Ekstrom and Ingelman-Sundberg, 1989). The THLE-2E1 cells (Mari and Cederbaum, 2000) are therefore already exposed to higher levels of oxidative stress compared with the THLE-Nulls resulting in the enhancement of defence mechanisms in the THLE-2E1 cells allowing them to survive better upon exposure to DEM compared with THLE-Null. This data also suggests that the trigger for the increase in ophthalmic acid is not or is only partly due to GSH depletion, but certainly involves the inability to up-regulate other cell defence pathways as in the THLE-2E1, these defence pathways would have already been up-regulated due to the higher levels of oxidative stress these cells are already exposed to prior to DEM treatment.

Treatment of Hepa1c1c7 with NAPQI demonstrated no noticeable change in GSH levels except for cells treated with 300 μM for 1 h (Figure 4.4). Furthermore, no change in ophthalmic acid levels was observed under all the treatment conditions used. Work within our group has previously demonstrated NAPQI to be a potent GSH depleter, with significant GSH depletion already demonstrated in Hepa1c1c7 cells after treatment with 3 μM NAPQI for 1 h compared with control (Copple *et al.*, 2008a). Such reactivity of NAPQI is evidently lacking in the present study and may have been due to degradation resulting in low reactivity of the NAPQI used. Due to time constraints, this could not be investigated further or repeated. However, where there was significant GSH depletion observed (cells treated with 300 μM for 1h, Figure 4.4 A), there was no corresponding increase in ophthalmic acid. Instead, ophthalmic acid was significantly elevated in cells treated with 300 μM NAPQI but 24 h later, when GSH was at control levels (Figure 4.4 B). Although this particular experiment requires repeating, the results indicated that ophthalmic acid is not a direct marker of GSH depletion.

Preliminary work carried out in the THLE cells with APAP (Figure 4.6) indicated GSH depletion in the THLE-2E1 cells compared with THLE-Null at 24 h. This difference is more prominent at 48 and 72 h with GSH depletion already observed at 15 mM APAP at these timepoints but was only apparent at 25 mM APAP at 24 h in the THLE-2E1 cell line. At the same time, a trend towards increasing ophthalmic acid was observed. These results, although preliminary, further suggests that other factors are involved that triggers the increase in ophthalmic acid.

Work presented in this chapter has demonstrated that ophthalmic acid is not a direct marker of GSH consumption in *in vitro* models but may still prove useful as a marker of oxidative stress when used in conjunction with other biomarkers. The disparity in ophthalmic acid response between the *in vitro* and *in vivo* experiments described in this chapter may be due to the model systems themselves. As cell lines are isolated systems, other extracellular factors or perhaps signalling between cell types are involved in the increase in ophthalmic acid. It is also possible that a different cell type to the cell lines employed here are responsible for the increase in ophthalmic acid synthesis, therefore *in vitro* studies would have to specifically use a certain cell type. The mechanism that triggers the increase in ophthalmic acid deserves further investigation as the consistent response in ophthalmic acid levels during chemically-induced oxidative stress may have biological significance that the cell employs to survive under such stress.

Chapter 5

Investigating the potential of ophthalmic acid as a translatable biomarker of chemical stress in the clinic

TABLE OF CONTENTS

5.1 INTRODUCTION

Potential biomarkers should satisfy a set of criteria that will determine whether they qualify as markers of a particular health condition or drug effect. Ideally, a biomarker should be organ specific, have strong correlation with other already existing well-defined markers of a specific injury, be sensitive in terms of timing (i.e. promptly indicative after an insult to allow physicians to intervene by withdrawing or introducing medication(s) for example, but also return to normal once the insult has been removed or the damage repaired) and detection (i.e. low but detectable basal levels with easily detectable increases), not subject to diurnal variation or diet-induced, adaptable for high throughput screening assays, detectable in biological matrices that can be accessed non-invasively (blood and/or urine), increase in levels and transferable across pre-clinical and clinical applications (Boone *et al.*, 2005, Ramaiah, 2007, Ozer *et al.*, 2008, Laverty *et al.*, 2010). However, a biomarker of chemical stress possessing all these attributes is yet to be discovered.

The work described in chapter 4 clearly indicates that ophthalmic acid is a potential biomarker of chemical stress. It was also markedly elevated in mouse liver following exposure to both acute and chronic treatment with APAP (chapter 3) and more importantly, this was reflected by a similar increase in serum, suggesting its utility as a minimally invasive marker of chemical stress. Similar results have been demonstrated in other laboratories (Soga *et al.*, 2006) where they also showed that the depletion of GSH by DEM produced the same increase in both hepatic and serum ophthalmic acid. Administration of BSO, an inhibitor of GCL, in contrast, resulted in a decrease in both hepatic and serum ophthalmic acid and GSH, demonstrating the similarities in their synthesis.

As such, the work described in this chapter explored the potential utility of ophthalmic acid as a translational clinical biomarker by establishing whether it is detectable and quantifiable in the serum of healthy individuals. Furthermore, this study also determined the intra- and inter-individual variation of ophthalmic acid and the effect this has on the potential of ophthalmic acid as a chemical stress biomarker. Ultimately, the aim was to explore the possibility of progressing ophthalmic acid as a clinical biomarker of chemical stress.

5.2 MATERIALS AND METHODS

5.2.1 Materials

Ophthalmic acid was purchased from Bachem (Weil am Rhein, Germany). Acetone (Normapur) and Formic Acid (AnalR NormaPur 99-100%) were both purchased from VWR (Lutterworth, Leicestershire, UK). Bovine Serum Albumin (BSA) and *S*-hexylglutathione were both purchased from Sigma Aldrich Chemical Co. (Poole, Dorset, UK). Methanol (HPLC Grade) and Infinity ALT (GPT) liquid stable reagent were purchased from Thermo Scientific (Loughborough, Leicestershire, UK).

5.2.2 Volunteers

The protocol employed for sample collection (section 5.2.3) and consent forms used for this study are under the approval of the Capenhurst Independent Research Ethics Committee (CIREC). The study group consisted of 5 male healthy volunteers with ages ranging between 20 and 54 years old, with an average age of 38 (± 11.56 SD). Characteristics of the volunteers are given in Table 5.1. This study aimed to determine the extent of normal ALT and ophthalmic acid variation therefore the volunteers were not subjected to any dietary restrictions such as alcohol consumption.

Table 5.1 Volunteer details

Volunteer	Age	Gender	Ethnicity	Smoker	Dietary limitations
1	46	Male	Caucasian	No	None
2	34	Male	Caucasian	No	None
3	28	Male	Caucasian	No	None
4	28	Male	Chinese	No	None
5	54	Male	Caucasian	No	None

5.2.3 Serum sample collection

Blood samples were collected from five male healthy volunteers for five consecutive days (days 1 – 5) and followed up with one collection per week for the next three weeks (days 8,

15 and 22). Blood samples were consistently taken at the same time on each day of collection. The blood samples were left to clot at room temperature for 30 minutes and centrifuged at 2600 g (SANYO MSE Harrier 15/80, Loughborough, Leicestershire, UK) for 20 minutes. Serum samples were carefully transferred into cryovials and stored at -80°C until required for analysis.

5.2.4 Ophthalmic acid analysis

5.2.4.1 Sample preparation

Each serum sample (10 µl) was spiked with 10 µl 5 µM *S*-hexylglutathione and made up to 50 µl with dH_2O. For the standards, 10 µl standard (to make final concentrations of up to 1 µM), 10 µl 5 µM *S*-hexylglutathione and 10 µl 30 mg/ml BSA were added together and made up to 50 µl with dH_2O. The internal standard *S*-hexylglutathione was prepared by making 1 mL of a 10 mM solution by firstly adding 450 µl 0.1 % formic acid and 50 µl 1 M NaOH to allow the *S*-hexylglutathione to dissolve into solution. A further 500 µl 0.1 % formic acid was added to give a concentration of 10 mM. Further dilutions to obtain 5 µM *S*-hexylglutathione were carried out with 0.1 % formic acid.

Samples and standards were de-proteinated with 200 µl ice-cold acetone, vortexed and left to precipitate at -20 °C for 1 hour. After de-proteination, the samples were centrifuged (Beckman GS-15R, High Wycombe, UK) for 10 minutes at 4 °C and the supernatants collected. Acetone used for de-proteination was removed by drying the supernatants down to approximately 50 µl in a speed vac (Eppendorf Concentrator 5301, Cambridge, UK) at 30 °C for 25 minutes. The concentrated standards were further diluted with 200 µl 0.1 % formic acid. Both neat serum samples and diluted standards were stored at 4 °C prior to analysis.

5.2.4.2 LC-MS-MS analysis

Samples were analysed on a QTRAP®5500 hybrid triple-quadrupole/linear ion trap instrument with Turbolon V™ Ion source (Applied Biosystems, UK) and were delivered into the instrument by automated in-line LC (Ultimate 3000 (Dionex, UK)), Gemini C18, 3 µm, 2.1 x 100 mm column (Phenomenex, UK)). A gradient from H_2O/0.1% Formic Acid (FA) v/v to 100% Acetonitrile/0.1% FA v/v in 15 min was applied at a flow rate of 200 µL min^{-1}. The QTRAP®5500 was operated in positive ESI mode with the following parameters optimised; ionspray potential 5500 V; nebuliser gas (GAS1) 50 psi; auxiliary gas (GAS2) 60 psi; ion spray heater to 700°C and medium collision gas. Two MRM transitions were monitored for both ophthalmic acid (290/58 and 290/161.1) and the internal standard (*S*-hexylGlutathione – 0/247.1 and 0/162.1) with a 100 ms dwell time. The declustering potential, focusing potential, collision energy, cell entrance potential and cell exit potential were optimised for each MRM transition.

5.2.5 ALT activity assay

Serum samples were collected and prepared as described in sections 5.2.2 - 3 and ALT levels were determined using Infinity ALT (GPT) reagent (Thermo Scientific, Loughborough, Leicestershire, UK) according to the manufacturer's instructions. Samples analysed for ALT activity were the same samples analysed for basal ophthalmic acid levels.

5.2.6 Statistics

Intra- and inter individual variation of ophthalmic acid level and ALT activity were analysed by calculating the mean concentration/activity, standard deviation (SD) and coefficient of variation (%). Low SD and CV values relative to the relevant data set were determined to have low dispersion of data points and high reproducibility, and vice versa.

5.3 RESULTS

5.3.1 Detection and quantification of basal ophthalmic acid in human serum

In order to compare the relative concentration of ophthalmic acid in rodent and human blood, serum samples from mouse, rat and man were analysed by mass spectrometry using both the in-house instrumentation described in chapter 2 and using a state-of-the-art triple quadrupole ion-trap instrument (AB Sciex QTrap 5500) in the laboratory of AB Sciex. Whilst mouse and rat plasma ophthalmic acid was measurable using the API3000 mass spectrometer, no ophthalmic acid was detectable in human serum under the same conditions. However, it was possible to detect and quantify ophthalmic acid in human samples using the QTRAP5500. Comparative basal ophthalmic acid levels for the three species are shown in Table 5.2 and the MRM mass spectra for each is given in Figure 5.1. It can be seen from Table 5.2 that the resting plasma levels of ophthalmic acid are approximately 20 – 30 times lower in man than in mouse, necessitating the use of a highly sensitive mass spectrometer for detection. The basal value obtained in rat (0.007 nM) was intermediate between man and mouse. No differences in the levels of ophthalmic acid were observed between male and female mice, nor when C57BL/6 mice were compared with levels in CD1.

Table 5.2 Basal ophthalmic acid levels in the serum of mouse, rat and man.

Sample ID	Sample type	Ophthalmic acid (nM)
A	Human - Male	0.002
B	Mouse – Male C57BL/6	0.054
C	Mouse – Female C57BL/6	0.060
D	Mouse – Male CD1	0.045
E	Rat – Male Wistar rat	0.007

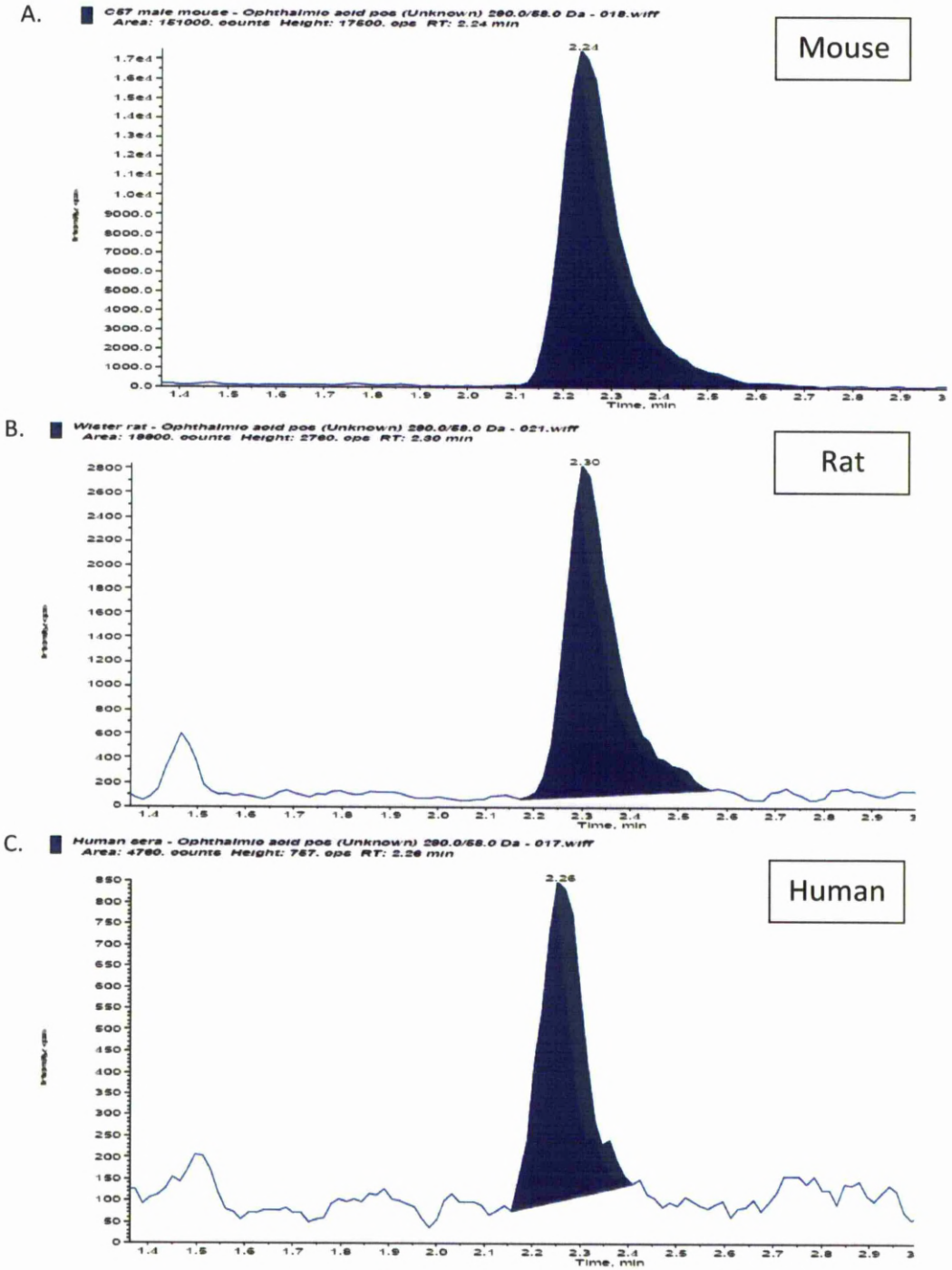


Figure 5.1 Basal ophthalmic acid in male (A) C57Bl6 mouse, (B)Wistar rat and (C) human serum using the AB Sciex QTrap 5500 LC-MS-MS system. The spectra show the MRM peaks for ophthalmic acid in equivalent amounts of each serum sample. Note the difference in ion count scale on the Y-axis, indicating the low levels found in human relative to rodent samples. Mean values derived from the analysis are given in Table 5.2.

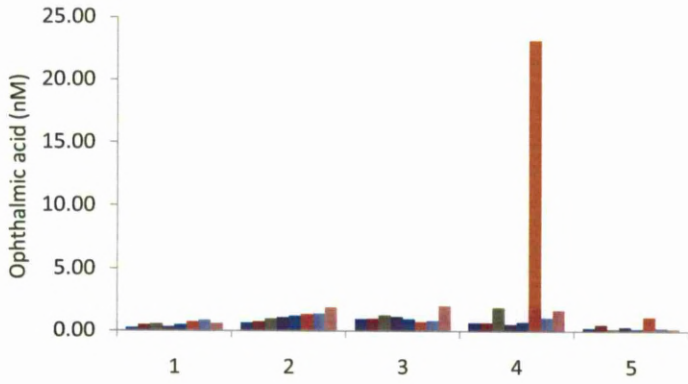
5.3.2 Intra-individual variation of basal ophthalmic acid and ALT activity in human serum

In order to define the inter- and intra-individual variation in basal ophthalmic acid levels, serum was collected from five volunteers at specified times up to four weeks (Figure 5.2) and both ophthalmic acid and ALT levels were measured. The mean concentration of basal ophthalmic acid from each volunteer ranged between 0.37 nM to 1.17 nM, with the lowest value observed from volunteer 1 and highest from volunteer 3. This range in mean concentration extended to a maximum of 3.81 nM when data from volunteer 4, day 8 (V4:8) was included since this was measured at about 10 times greater than all other values, and was thus considered an atypical value (Figures 5.2 A and C).

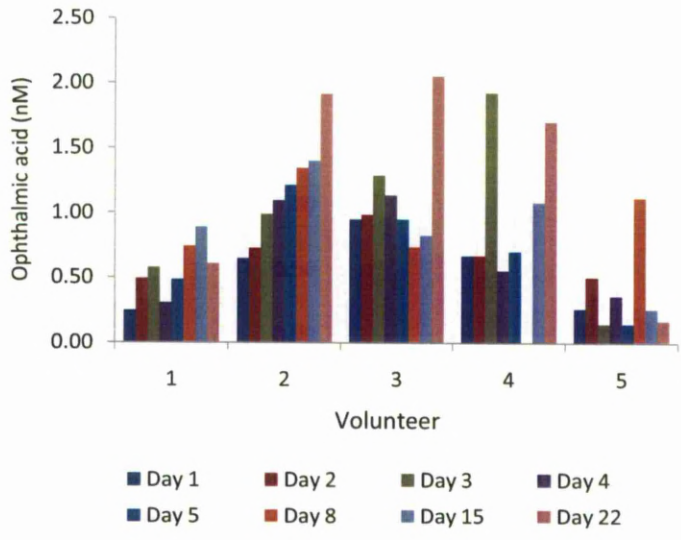
The SD (nM) was calculated for each volunteer in order to determine the profile of ophthalmic acid concentration across the 8 days. The SD values ranged between 0.21 nM – 0.56 nM, or up to 7.84 nM when including V4:8. These values indicate that ophthalmic acid levels vary between individual and within an individual (Figure 5.2 C).

In order to assess if the degree of variation is similar between volunteers, the CV (%) was calculated. The CV values ranged between 34.89 % - 88.11 %, or up to 205.92 % when including V4:8. Volunteers 1 – 3 shared a similar degree of variation (34.89 % - 39.07 %) in ophthalmic acid levels around their respective mean concentrations. Levels from volunteers 4 (53.47 %) and 5 (88.11 %) tended to be more variable, especially when data from V4:8 (205.92% for volunteer 4, Figure 5.2 C) was taken into account.

A



B



C

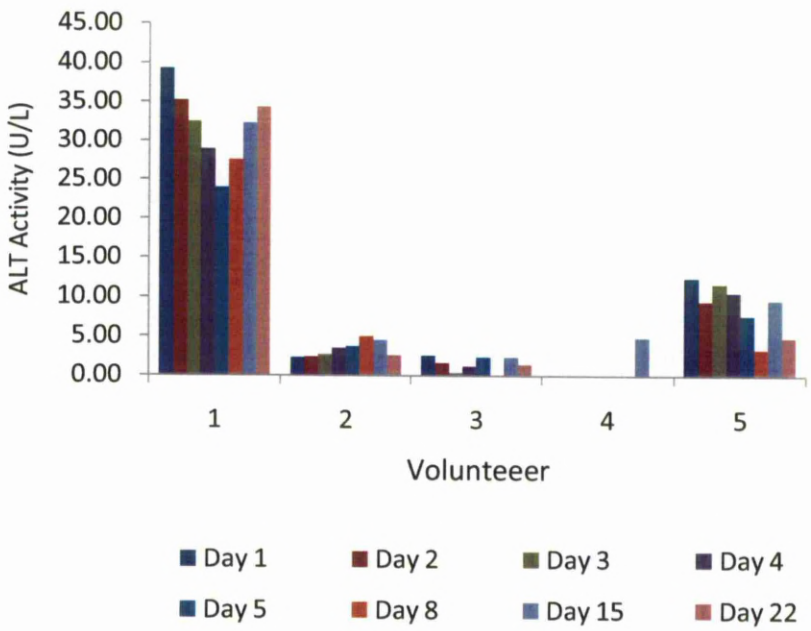
Volunteer	Mean (nM)	SD (nM)	CV (%)
1	0.54	0.21	39.07
2	1.17	0.41	34.89
3	1.11	0.42	37.44
4	1.04	0.56	53.47
4[a]	*3.81*	*7.84*	*205.92*
5	0.37	0.33	88.11

Figure 5.2 Intra-individual variation of basal ophthalmic acid in the serum of 5 male healthy volunteers. Blood samples were collected for 5 consecutive days (days 1 – 5) and followed up by one day per week for the next three weeks (days 8, 15 and 22). Serum was collected from each blood sample as detailed in sections 5.2.2-3 and analysed for ophthalmic acid by LC-MS-MS as described in section 5.2.4. **A** represents ophthalmic acid data including V4:8, an outlier highlighted as *4[a]* in C. **B** represents ophthalmic acid data excluding the outlier (*4[a]*). **C** summarises the mean concentration (nM), standard deviation (SD nM) and coefficient of variation (CV %) of ophthalmic acid concentrations of all 8 visits from each volunteer.

ALT activity was measured at each time point and is presented in Figure 5.3. Every value in all the volunteers was below the upper limit of normal (defined as 40 U/L), however, considerable variation was observed between individuals. Volunteer 1 consistently had values that were just below the ULN whereas in volunteer 4, ALT activity could only be detected at day 15. Overall, whilst considerable inter-individual variation is observed, values within an individual were relatively consistent.

The CV values for ALT activity ranged between 15.2 % - 62.9 %, with the lowest value observed from volunteer 1 and the highest from volunteer 3. Only one ALT value was obtained from volunteer 4, consequently no estimate of variance was possible.

A.



B.

Volunteer	Mean (U/L)	SD (U/L)	CV (%)
1	31.76	4.81	15.15
2	3.33	1.04	31.26
3	1.48	0.93	62.86
4	4.83	N.D.	N.D.
5	8.69	3.22	37.03

Figure 5.3 Intra-individual variation of ALT activity in the serum of 5 male healthy volunteers. Blood samples were collected for 5 consecutive days (days 1-5) and followed up by one day per week for the next three weeks (days 8, 15 and 22). Serum was collected from each blood sample as detailed in sections 5.2.2-3 and analysed for ALT as described in section 5.2.5. **A** represents ALT activity levels (U/L). **B** summarises the mean concentration, standard deviation (SD) and coefficient of variation (CV) of ALT activity levels in all 8 visits for each volunteer.

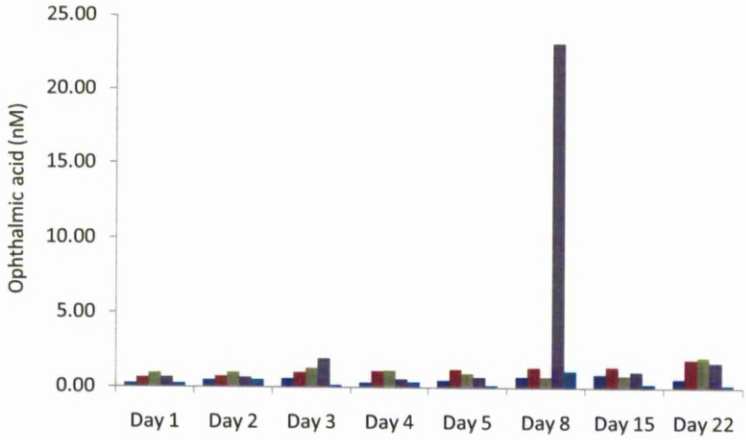
5.3.3 Inter-individual variation of ophthalmic acid and ALT activity in basal human serum

The mean concentration range of ophthalmic acid across all five volunteers was between 0.55 nM – 1.29 nM. However, the maximum concentration extended to 5.42 nM when all data from volunteer 4 were taken into account (Figures 5.4 A – B).

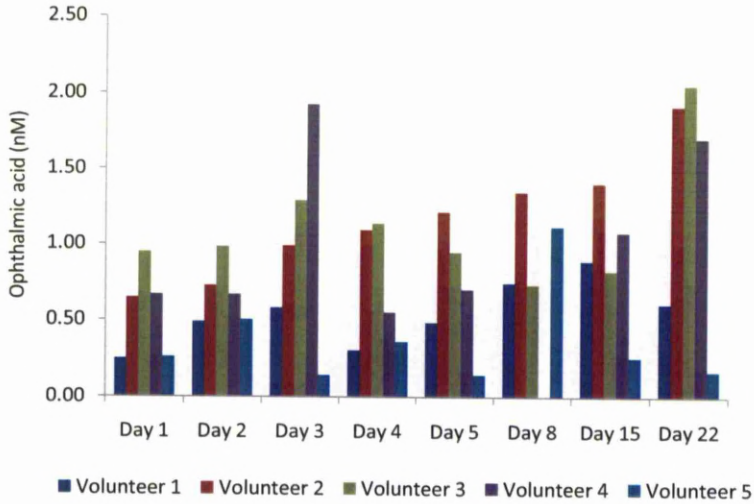
SD values were also calculated in order to determine the spread of ophthalmic acid concentration from each day. The SD values ranged between 0.2 nM – 0.85 nM, or up to 9.92 nM from V4:8 (Figure 5.4 C), very similar to observations made from the intra-individual variation analysis.

The mean ALT activity range was between 7.19 (day 8) – 11.30 (day 1) U/L, which again was below the ULN of 40 U/L. SD values were calculated with the least varied observed on day 5 (9.6 U/L) and the most varied observed on day 1 (16.36 U/L). The CV values that ranged between 115.17 % - 167.71 %, were in general of high values but were comparable between visits with the least variable set of data observed on day 15 and the most variable observed on day 22. The high CV values observed from this analysis demonstrated the high degree of variability in ALT activity between individuals (Figures 5.5 A – B).

A



B

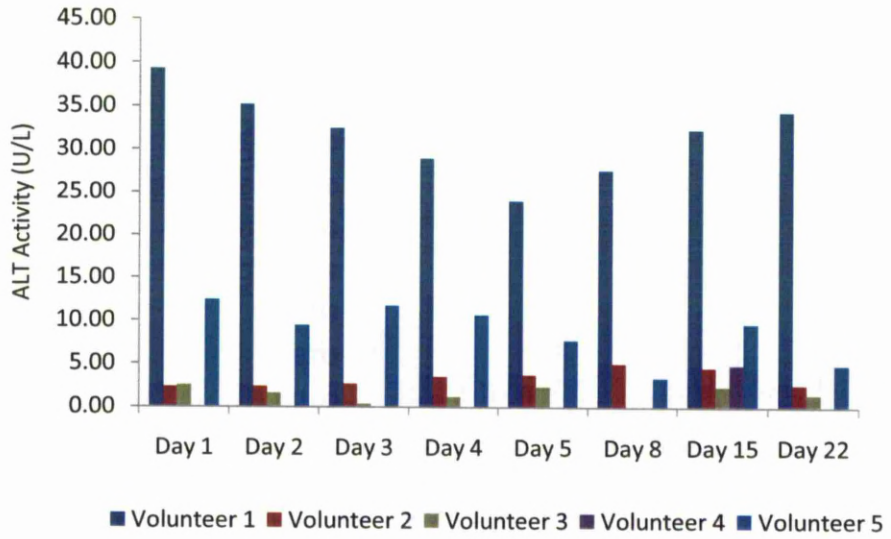


C

Visit (Day)	Mean (nM)	SD (nM)	CV (%)
1	0.55	0.30	53.90
2	0.67	0.20	29.92
3	0.98	0.68	69.10
4	0.69	0.40	57.99
5	0.70	0.41	59.06
8	0.79	0.51	57.99
8[a]	*5.42*	*9.92*	*183.09*
15	0.89	0.42	47.08
22	1.29	0.85	65.61

Figure 5.4 Inter-individual variation of basal ophthalmic acid in the serum of 5 male healthy volunteers. Blood samples were collected for 5 consecutive days (days 1 – 5) and followed up by one day per week for the next three weeks (days 8, 15 and 22). Serum was collected from each blood sample as detailed in sections 5.2.2-3 and analysed for ophthalmic acid by LC-MS-MS as described in section 5.2.4. **A** represents inter-individual variation of ophthalmic acid concentration including V4:8, an outlier highlighted as *8[a]* in C. **B** represents the same data to A, excluding *8[a]*. **C** summarises the mean concentration, standard deviation (SD) and coefficient of variation (CV) of ophthalmic acid concentration between the five volunteers.

A



B

Visit (Day)	Mean (U/L)	SD (U/L)	CV (%)
1	11.30	16.36	144.70
2	9.72	14.69	151.20
3	9.44	13.73	145.55
4	8.83	11.96	135.37
5	7.54	9.60	127.32
6	7.19	11.59	161.06
7	10.72	12.35	115.17
8	8.64	14.49	167.72

Figure 5.5 Inter-individual variation of ALT activity in the serum of 5 male healthy volunteers. Blood samples were collected for 5 consecutive days (days 1 – 5) and followed up by one day per week for the next three weeks (days 8, 15 and 22) (Table 5.1). Serum was collected from each blood sample as detailed in sections 5.2.2-3 and analysed for ALT as described in section 5.2.5. **A** represents ALT activity levels (U/L). **B** summarises the mean concentration, standard deviation (SD) and coefficient of variation (CV) of ALT activity levels between the five volunteers.

5.4 DISCUSSION

At present, many biomarkers are in use for preclinical and clinical applications despite shortcomings with regards to their specificity, accessibility and transferability (for a review on liver biomarkers, please refer to Ozer (2008). ALT for instance is widely used as a principal biomarker for liver injury although elevations have been reported in cases other than (Muller *et al.*, 2000), or with no evidence of, liver injury (Jackson *et al.* 2008). Furthermore, intrinsic factors such as gender, age and body mass index (BMI) (Kariv *et al.* 2006) as well as polymorphic differences (Yuan *et al.* 2008) have been shown to influence serum ALT levels. As such, there is a pressing need for new biomarkers to support the current panel by further substantiating toxicity profiles used to characterise various liver conditions. Presently, the discovery of a number of potentially useful liver biomarkers that have been reported in the literature, a list of which is summarised in chapter 1, Table 1.6 merit further investigation. These include α-glutathione-*S*-transferase (MacGregor 2003), cytochrome c (Miller *et al.* 2008), regucalcin (Yamaguchi *et al.* 2002), arginase 1 (Ashamiss *et al.* 2004, Murayama *et al.* 2008), ornithine carbamyl transferase (Amacher 2002, Murayama *et al.* 2008) , paraoxonase (Amacher *et al.* 2005), ALT isoforms (Miyazaki 2009), calpain (Limaye *et al.* 2003), sPLA2 (Bhave *et al.* 2008), HMGB1 (Antoine *et al.*, 2009) and liver-specific isoforms of ALP (Amacher *et al.* 1987), all of which are potentially useful as markers of hepatotoxicity.

The study presented in this chapter explores the potential of ophthalmic acid as a biomarker for chemical stress in the clinic. Interest in ophthalmic acid arose from observations made initially by Soga *et al.* (2006) and subsequently repeated in our own laboratory (chapter 4), where ophthalmic acid levels were significantly elevated in the liver and serum of APAP-treated mice in parallel to a significant depletion in hepatic GSH. This correlation has brought attention to the potential role of ophthalmic acid as a surrogate marker of GSH consumption in response to chemical stress, which is of significance given the important role of GSH in the detoxification of both electrophiles and reactive oxygen species during cellular stress (chapter 1). GSH itself does not have the potential to be a non-invasive biomarker of chemical stress, an important feature of a clinical biomarker, since its stability outside the cell is low due to the instantaneous degradation of GSH through the activity of gamma-glutamyl transpeptidases, allowing reabsorption of the amino acid constituents of GSH in tissues for various cellular processes (Meister and Anderson 1983; Meister 1984). Furthermore, although it is routinely used as a chemical stress biomarker in

preclinical studies, the oxidative susceptibility of the thiol group of GSH does not make it amenable to adapt to current clinical protocols and would require careful consideration with regards to sample handling if accurate measurements were to be achieved. The nature of its function as a detoxification molecule, directly or indirectly, during chemical stress leads to its depletion which could affect the level of sensitivity in which it could be measured. On the other hand, a biomarker such as ophthalmic acid which does not only demonstrate a potentially important function but is also more chemically stable, at least during measurement in the laboratory, due to the replacement of the sulphydryl-containing cysteine in the GSH molecule with 2-AB where the sulphydryl group is replaced by a methyl group, deserves further investigation in both preclinical and clinical applications. Unlike GSH, an increase in ophthalmic acid levels can be measured in response to cellular stress indicating a potentially sensitive marker of GSH consumption.

As demonstrated in the results section of this chapter, the detection and quantification of basal ophthalmic acid in human serum was established through the use of a highly sensitive LC-MS-MS system. This present study along with that carried out by Kombu *et al.* (2009), where they investigated the rate of ^{2}H labelling of GSH, GSSG and ophthalmic acid from ^{2}H-enriched body water, are the first to report the presence of ophthalmic acid in human serum. Furthermore, the concentrations observed, with 0.14 nM being the lowest concentration detected across all the serum samples, are within the ideal concentration range of a serum biomarker at basal level, with the exception of one determination. As such, the presence of ophthalmic acid in human serum and the capability to detect and quantify it provides a platform to further investigate the translatability of ophthalmic acid as a clinical biomarker of chemical stress. However, although this has provided positive results, only a small sample size was used to carry out this study therefore an extension of this work using a larger population size is required.

The intra- and inter-individual variability of ophthalmic acid was also investigated from the same sample group. The degree of inter- and intra-individual variation of ophthalmic acid differed amongst the participants. However, apart from a sample collected from volunteer 4 on day 8, all ophthalmic acid concentrations measured were below 2 nM. The ALT activity levels were also analysed from the same samples as a comparator biomarker. Similar to observations made with ophthalmic acid, there were also differing degrees of inter- and intra-individual variation in ALT activity amongst the participants however, all values were below the ULN. This suggests that under normal physiological conditions, there will always be intrinsic and extrinsic differences in ophthalmic acid

concentration and ALT activity levels within and between healthy individuals. With regards to ophthalmic acid, the key is to determine the absolute maximum level in human serum that is indicative of a healthy individual, similar to how the ULN was determined for ALT activity (Hy's Law) (Zimmerman, 1968, Zimmerman, 1978, Senior, 2009) which is at present, routinely used as guidance for diagnosing hepatotoxicity in the clinic.

Data from volunteer 4 displayed the highest concentration of ophthalmic acid in this study which was interestingly followed a week later by the only ALT measurement obtained from this volunteer on day 15. All participants were assumed to be healthy at the start and throughout the course of this study. However, it should be noted that they were not restricted to a particular lifestyle such as following a set diet regimen as the aim of this study was to encapsulate the degree of variability of ophthalmic acid in a healthy population with differing lifestyles. As such, the high ophthalmic acid level on day 8 and the ALT activity measured on day 15 from volunteer 4 may have been triggered by a change in the lifestyle activity of volunteer 4. For example, a change in alcohol intake, vigorous exercise or new medication could account for the above changes despite the absence of overt liver injury. Furthermore, volunteer 4 was the only volunteer from a different ethnic background which may reflect genetic differences between ethnic populations. Intrinsic factors such as gender and polymorphic differences have been demonstrated to affect basal ALT levels (Kariv *et al.*, 2006, Yuan *et al.*, 2008), and this would be expected for other potential markers, such as ophthalmic acid. It is therefore likely that the above factors may have the potential to perturb levels of biomarkers such as ophthalmic acid.

Although the above observation was only made from one individual, this could be indicative of a correlation between ophthalmic acid and ALT. As the increase in ophthalmic acid preceded the observed increase in ALT, this may suggest that ophthalmic acid is an early biomarker of chemical stress. This follows a similar trend to that observed in chapters 3 and 4 in the serum of mice treated with APAP where the increase in ophthalmic acid also preceded the increase in serum ALT. The sensitivity of this correlation was demonstrated by the change in both ophthalmic acid and serum ALT in an individual who was assumed to be healthy as there was no report of any change in this person's health throughout the study. This observation is critical in the design of future experiments, in particular when investigating whether observations made in chapters 3 and 4 and by Soga *et al.* (2006) in mice can also be demonstrated in humans. For this work, it is important to determine the effects of APAP both at therapeutic and toxic levels in order to determine the degree of change in ophthalmic acid.

Biomarkers are known to be influenced by several factors which may influence the basal and inducible levels of a biomarker, as well as the degree of response observed in a biomarker following a given stimulus. Such factors include gender, age, BMI and polymorphic differences, all of which have been shown to affect plasma ALT activity (Kariv *et al.* 2006; Yuan *et al.* 2008). Investigating the variability of basal ophthalmic acid with regards to such factors, in addition to those discussed earlier, would therefore be of benefit in establishing whether a standardised basal concentration can be used as a point of reference by all future users, or whether such standard concentrations should be set categorically depending on certain variables that have been previously shown to have an impact on other biomarkers. Indeed as mentioned earlier, difference in ethnic background may be an influencing factor in ophthalmic acid levels as volunteer 4 who is from a different ethnic background compared with the other volunteers displayed the highest ophthalmic acid measured in the study.

Several studies have also explored the distribution of ophthalmic acid in different species (Waley, 1958, Reddy *et al.*, 1966, Takanori Kasai and KIRIYAMA, 1989a, Takanori KASAI and KIRIYAMA, 1989b, Soga *et al.*, 2006). However, as the function of ophthalmic acid is unknown, the physiological significance of variability in tissue distribution and abundance across species is not yet understood. Interestingly, not all previous distribution studies have reported ophthalmic acid to be detected in the liver. This suggests highly varied levels of hepatic ophthalmic acid among species even down to the extreme of undetectable levels. However, this variability in hepatic ophthalmic acid may reflect a lack of sensitivity in the assay. The presence of hepatic ophthalmic acid across preclinical models and in humans would be of great significance as the liver is a major site of drug metabolism and is therefore also a major target for xenobiotic-induced stress and toxicity.

Although the distribution and abundance of ophthalmic acid has been established in several tissues, only a few groups have reported the presence and concentration of basal ophthalmic acid in serum (Kombu *et al.* 2009; Soga *et al.* 2006). This chapter describes basal levels of ophthalmic acid in mouse, rat and human serum. Ophthalmic acid was found to be most abundant in mouse followed by rat and then human sera (Figure 5.2). Preliminary work was also carried out in the department to determine whether ophthalmic acid levels were influenced by diurnal variation in rodents (data not shown), however, no effect of the time of sampling within a 24 h period was observed. As discussed earlier, the accessibility of a biomarker, especially in biofluids, is crucial in assessing the transferability of a biomarker from preclinical to clinical use. Although basal serum ophthalmic acid levels are different

amongst species, its consistent presence in the serum advances its potential role as a transferable biomarker for chemical stress. Similar findings were observed in current biomarkers such as ALT where basal levels differ between species and organ sources (Lindblom *et al.* 2007; Miyazaki 2009; Rajamohan *et al.* 2006). As such, in order for ophthalmic acid to be used as a sensitive biomarker of chemical stress in preclinical and clinical applications, differences in its basal concentration between models systems should be determined.

In summary, the experiments detailed in this chapter, which represent the first systematic attempt to define ophthalmic acid baseline characterisation in man, suggest that ophthalmic acid may have potential for clinical use. The serum concentrations of ophthalmic acid at basal levels are very low in man, compared with mice which whilst a desirable property, poses problems bioanalytically. Ophthalmic acid was only detectable using the AB Sciex 5500 QTRAP mass spectrometer which is currently one of the most sensitive instruments on the market. Quantification of analytes at levels approaching the lower limit of detection is likely to lead to loss of precision through analytical error. Nevertheless, the work described in this chapter indicates that ophthalmic acid levels are generally stable within a given individual and that variation between individuals is similar to that observed with the established biomarker of liver damage, ALT. Furthermore, the high level of ophthalmic acid detected in a single sample from one of the volunteers (V4:8), whilst unexplained, does suggest a significant dynamic range for ophthalmic acid in response to a stress stimulus. Overall, the data described here are supportive of further investigation of ophthalmic acid as a clinically applicable biomarker of chemical stress in larger volunteer cohorts. Subsequently, its qualification as a biomarker should be assessed in patients exposed to a known chemotoxic stimulus, such as APAP overdose.

6. FINAL DISCUSSION

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6.1 Introduction

Today, public health is heavily dependent on the use of drugs for the cure and management of a wide array of health conditions. Ironically however, they may also pose threat to public health when administered incorrectly or due to an idiosyncratic reaction. Such threats can be a consequence of inadequacies in the research of a drug as further work is often hampered by the unavailability of appropriate tools as well as the complexity of the pharmacology of a drug.

Research tools that are able to accurately simulate the pharmacology of a drug in a human system would be highly advantageous for the investigation of the mechanistic basis of ADRs and as preclinical screening systems for the assessment of toxicological potential. As such, a great deal of research is in progress for the development of better model systems in an attempt to reduce the high attrition rate of new drug candidates. The work carried out during this PhD project was aimed at the identification of development of new model systems for investigating the consequences of chemical stress in the liver. The development of such model systems will allow the investigation of both the toxicological consequences of drug-induced stress and also the adaptive responses that have evolved within mammalian systems to provide defence against noxious chemical stimuli. Ultimately, a better understanding of these processes afforded by the use of these model systems should result in the identification of new biomarkers of value as preclinical tools or translational indices of chemical stress.

Two independent models were characterised: first, the use of a cell line expressing cytochrome P450 shown to generate oxidative stress, and secondly, a transgenic mouse model deficient in a major regulator of the stress defence response, Nrf2. In addition, the GSH analogue, ophthalmic acid, levels of which have been demonstrated to increase, in concurrent with GSH depletion, was assessed for its potential as an oxidative stress biomarker.

6.2 Exploiting the cytochrome P450 enzyme family as models of chemical stress.

As detailed in chapter 1, the CYP450 enzyme family plays an important role during phase I metabolism. Consequently, they are also responsible for the bioactivation and subsequent accumulation of reactive species that may overwhelm cellular defence system potentially leading to adverse drug reactions. Furthermore, polymorphisms in these enzymes may influence an individual's susceptibility to such reactions (Morgan *et al.*, 1984, Alvan *et al.*, 1990, Pirmohamed and Park, 2001, Nakamura, 2008). As such, exploitation of this enzyme family in understanding xenobiotic-induced stress has been well exercised through the employment of various methods such as the manipulation of CYP450 protein expression in both *in vitro* (Mace *et al.*, 1998, Bull *et al.*, 2001, Dambach *et al.*, 2005) and *in vivo* (Gonzalez, 2002, Cheung and Gonzalez, 2008) systems. The establishment of such systems have in turn been applied in drug metabolism studies as well as for understanding the biological consequences of altered protein expression, which in turn provides insight into the metabolic fate of a drug under these conditions.

Although the use of primary hepatocytes is the preferred method for studying drug-induced chemical stress, limitations of this model as detailed in chapter 1, particularly in its (rapid) loss of metabolic capacity soon after isolation impedes its practical use in research. As such, immortalised cell lines which have been manipulated to overexpress individual CYP450 isoforms have been adopted as surrogates of primary hepatocytes. Many such cell lines already exist (Dai *et al.*, 1993, Bull *et al.*, 2001) however, the THLE CYP450 over-expressing cells are the first to be developed as a panel of cell lines originating from one parental source providing direct comparators for a given study (Pfeifer *et al.*, 1993, Mace *et al.*, 1998, Dambach *et al.*, 2005). As part of this PhD project, the THLE-2E1 cell line was characterised and investigated for its utility as a model of chemical stress due to the CYP2E1 protein's metabolic role in the bioactivation of a number of clinically relevant drugs/compounds with APAP (Morgan *et al.*, 1983, Raucy *et al.*, 1989, Lee *et al.*, 1996, Zaher *et al.*, 1998, Manyike *et al.*, 2000) and ethanol (Lieber *et al.*, 1970, Koop *et al.*, 1982, Ryan *et al.*, 1986, Bradford *et al.*, 2005) being well known examples, and due to the generation of ROS through its interaction with the CPR enzyme (Gorsky *et al.*, 1984, Ekstrom and Ingelman-Sundberg, 1989, Dai *et al.*, 1993, Mari and Cederbaum, 2000, Gong and Cederbaum, 2006).

Work carried out in the THLE-2E1 cells demonstrated CYP2E1 overexpression that was comparable with an equivalent amount of cryopreserved primary human hepatocytes. Furthermore, this CYP2E1 protein over-expression correlated with a distinct CYP2E1 metabolic activity that was assessed and demonstrated through the use of two well known CYP2E1 substrates, APAP and chlorzoxazone. As such, this demonstrates that from a metabolism point of view, the THLE-2E1 cells could be useful in metabolism studies of new and existing drugs. However, further validation of this utility would have to be carried out through full metabolic characterisation of other well established drugs known to be principally metabolised by the CYP2E1 enzyme.

The ability of CYP2E1 to produce high levels of ROS species due to its loose coupling with CPR has been reported in a number of model systems (Gorsky *et al.*, 1984, Ekstrom and Ingelman-Sundberg, 1989, Dai *et al.*, 1993, Mari and Cederbaum, 2000, Gong and Cederbaum, 2006). Through the measurement of GSH where levels were elevated in untreated THLE-CYP2E1 cells compared with the THLE-Null, also demonstrated higher levels of oxidative stress in the THLE-CYP2E1 cells at least in comparison to the THLE-Null cells, indicating the potential role of the THLE-2E1 cell line as a model of oxidative stress. Further insight into the adaptation mechanisms employed by the THLE-2E1, which could potentially indicate adaptative mechanisms in the liver during oxidative stress, could be obtained through further research. In particular, the increased GSH level in these cells suggests a role of the transcription factor Nrf2, being a transcriptional regulator of GSH synthesis, in the adaptation of the THLE-2E1 cells to oxidative stress. Indeed this was demonstrated in another CYP2E1 overexpressing cell line, HepG2-2E1 where the authors associated this increase in GSH with Nrf2 up-regulation. This therefore opens the possible role of Nrf2 in adaptation to oxidative stress not just in the GSH synthetic pathway, but also in other pathways in which other cytoprotective genes that it regulates are involved in. This could be further explored by measurement of Nrf2 levels in the THLE-2E1 cells versus the THLE-Null as well as target genes that Nrf2 has been reported to regulate. As GSH levels are clearly higher in the THLE-2E1 compared with the THLE-Nulls, measurement of GCL and GS protein and activity levels would be a good starting point for this further work.

Within the remit of the aims in this PhD project, the utility of the THLE-2E1 cell line may be limited to drug metabolism and basal oxidative stress studies, as chemical-induced toxicity was not detected under the experimental conditions used with APAP. This as discussed in chapter 2, could be a consequence of the adaptation of this cell line in response to higher levels of basal oxidative stress compared with the THLE-Nulls. The suggested role of Nrf2 in

this adaptation in the previous paragraph based on higher GSH levels in the THLE-2E1 cells compared with the THLE-Null may also mean up-regulation of other cytoprotective genes such as GSTs (Itoh *et al.*, 1997, Hayes *et al.*, 2000, Reisman *et al.*, 2009d) and MRPs (Maher *et al.*, 2005, Maher *et al.*, 2007). Various defence mechanisms may therefore be in play to defend basal and chemical-induced stress. As such, it would be difficult to study drug-toxicity in these cells particularly when defence systems may have already been up-regulated prior to treatment. Furthermore, although CYP2E1 activity was observed in the THLE-2E1 cells and not in the THLE-Nulls, this level of activity when compared with CYP2E1 activity in primary human hepatocytes as reported by Lahoz (2007) does not demonstrate the same magnitude of increase in activity as has been reported in the other CYP450 overexpressing THLE cell lines. As this is a comparison between two independent experiments from two independent laboratories, it would be ideal to carry out direct comparisons with regards to CYP450 activity in each of the CYP450 overexpressing cell lines, the THLE-Null cells and primary human hepatocytes to have a more accurate indication in the difference in CYP450 activities between these systems. However, based on this current comparison, although the THLE-2E1 may have a higher CYP2E1 activity when compared with what was reported by Lahoz (2007) in primary human heptocytes, this may not be sufficient to cause toxicity. Indeed, in normal APAP metabolism, only a small amount of the reactive metabolite NAPQI is produced, which is subsequently detoxified through GSH reaction. A certain amount of NAPQI is required for APAP-induced toxicity to take place, but this was not observed in our studies despite using a concentration of up to 25 mM APAP, demonstrating that CYP2E1 activity in these cells are insufficient to produce enough NAPQI to cause toxicity. This, in addition to the potentially enhanced defence system in the THLE-2E1 cells impede the utility of this cell line for imvestigating chemical-induced liver toxicity. Furthermore, despite the convenience of immortalised cell lines such as the THLE-2E1, care should be exercised when using models such as this as their establishment as immortalised cells and manipulated systems no longer reflect the physiological characteristics of an intact liver.

6.3 The role of Nrf2 in the GSH system: The utility of the Nrf2 knockout mouse as a model of chemical stress

As detailed in chapter 1, the regulatory role of Nrf2 in the basal and inducible transcription of a wide array of cytoprotective genes, including GCL, the enzyme responsible for the rate limiting step in GSH synthesis, brings Nrf2 to the forefront of cellular defence. As the GSH synthetic pathway is closely linked to several other metabolic pathways, referred to in this PhD project as the GSH system (chapter 1), the role of Nrf2 within the GSH system was investigated in response to chemical-induced stress. In particular, treatment of $Nrf2^{(+/+)}$ and $Nrf2^{(-/-)}$ animals with three different dosing regimens of APAP was carried out and investigated for the role of Nrf2 on the GSH system. In brief, $Nrf2^{(+/+)}$ and $Nrf2^{(-/-)}$ mice were administered with either: 1) an acute sub-toxic dose of 350 mg/kg across a 5 h time point; 2) escalating doses (0 – 600 mg/kg) over an 8 day period; or 3) escalating doses (0 – 600 mg/kg) over an 8 day period followed by a toxic dose challenge of 1000 mg/kg on day 9. In parallel, serum ALT activity was also measured in these animals to determine toxicity and to elucidate whether correlations can be established between toxicity and perturbations within the GSH system as a result of APAP treatment and Nrf2 deletion.

The use of the $Nrf2^{(-/-)}$ mouse in various laboratories has revealed the importance of this transcription factor in cell defence. In particular, the enhanced susceptibility of this model to chemical stress in comparison to its wild type counterpart has been well established with a variety of chemical stress inducers as detailed in chapter 1 (Chan *et al.*, 2001, Enomoto *et al.*, 2001, Umemura *et al.*, 2006, Xu *et al.*, 2008, Jiang *et al.*, 2009). As such, the work carried out in this project was performed to elucidate whether this susceptibility was due to Nrf2 deletion, which may have in turn affected the GSH system. Indeed findings by our group are consistent with reports in the literature of a 20 – 30 % decrease in hepatic GSH in the $Nrf2^{(-/-)}$ animals basally (Kitteringham *et al.*, 2010). The aim was to explore this further by also measuring perturbations within the GSH system, in addition to the decrease already observed in the basal GSH content of the $Nrf2^{(-/-)}$ animals.

Interestingly, the acute APAP study indicated that up to a certain level of chemical stress, both strains responded in a similar manner as demonstrated by the level of serum ALT up to and including the 3 h time point (chapter 3, Figure 3.3) observed. However, at 5 h, a distinct difference in response was observed as a further increase in serum ALT was observed in the $Nrf2^{(-/-)}$ APAP-treated animals but not in the wild type equivalents, when compared with their respective control groups. This suggests that the effect of Nrf2

deletion is only apparent when a certain threshold of chemical stress is reached. This agrees with the corresponding GSH data where recovery of hepatic GSH back to basal levels was more apparent in the $Nrf2^{(+/+)}$ animals than in the equivalent Nrf2 nulls. The ability to up-regulate GSH synthesis is therefore one factor that may have allowed the $Nrf2^{(+/+)}$ animals to attenuate further liver injury demonstrating the difference in response between the two strains.

Treatment with escalating doses of APAP (0 – 600 mg/kg) led to an increase in serum ALT in the $Nrf2^{(-/-)}$ animals whilst levels in the equivalent wild type remained comparable with their corresponding control further demonstrating the enhanced susceptibility of the $Nrf2^{(-/-)}$ animals to xenobiotic-induced stressed. It is interesting that although the dose range of APAP used during this treatment reached up to 600 mg/kg, a dose considered to be toxic to wild type animals when administered acutely, the wild type animals did not demonstrate toxicity in response to this treatment. Furthermore, although a marked increase in serum ALT was observed in the equivalent $Nrf2^{(-/-)}$ animals, the level of increase only indicated minimal hepatotoxicity. This demonstrates that although there is a distinct difference in response between the $Nrf2^{(+/+)}$ and $Nrf2^{(-/-)}$ animals to this treatment with regards to serum ALT, both strains were to some degree able to attenuate toxicity. This difference also suggests a role of Nrf2 in protection from such treatment, however, the lack of significant changes in the metabolites monitored within the GSH system suggests that this role does not necessarily involve modulations within the GSH system. Instead, other mechanisms could also be in play that have afforded both the wild type and $Nrf2^{(-/-)}$ animals protection from chronic APAP treatment, as will be discussed below.

Serum ALT and survival rate data for both $Nrf2^{(+/+)}$ and $Nrf2^{(-/-)}$ animals that were pretreated with saline or escalating doses of APAP and subsequently challenged with a toxic APAP dose (SA and AA groups respectively) further demonstrated the enhanced susceptibility of the $Nrf2^{(-/-)}$ animals to xenobiotic induced stress compared with the wild type. Interestingly, the AA group of both strains demonstrated a lower increase in serum ALT and better survival rate than the SA groups when compared with their respective controls. This suggests that paracetamol pretreatment afforded both strains protection against the toxic dose on day 9, the only difference being the $Nrf2^{(-/-)}$ animals were not able to afford the same level of protection as the wild type animals suggesting that this protection is in part Nrf2-mediated. However, similarities in response of both strains to the three treatment regimens with APAP indicate that Nrf2 is not the only factor involved in the attenuation and/or protection from xenobiotic-induced liver injury. Certainly with regards

to the two chronic dosing regimens, other factors could be involved as demonstrated by Shayiq and colleagues (1999) whereby they investigated the effect of chronic APAP treatment to a toxic dose challenge in mice. In this study, they reported autoprotection afforded by the APAP-pretreated animals against a subsequent toxic dose. This they reported to be due to a down-regulation in CYP2E1 and CYP1A2 activities, an increase in cell proliferation and therefore damage repair and a shift in APAP bioactivation from centrilobular to periportal regions where CYP2E1 was not found and where GSH was more abundant. As such, in addition to the role of Nrf2 by up-regulation of cell defence, particularly in GSH synthesis as discussed below, other adaptive mechanisms as reported by Shayiq and co-workers may have also been employed by both strains which could explain why similarities in response have been observed.

Perturbations within the GSH system observed in both strains under the three treatment regimens were generally comparable. Significant changes were consistently observed in GSH, GSSG and ophthalmic acid levels suggesting that these metabolites are the main players within the GSH system that respond to APAP treatment, although the other pathways in their own right are also important in cell defence. Although comparable patterns in metabolite levels were observed between the $Nrf2^{(+/+)}$ and $Nrf2^{(-/-)}$ animals, a delay in both GSH and ophthalmic acid synthesis was evident in the $Nrf2^{(-/-)}$ animals suggesting that the role of Nrf2 within the GSH system is focussed on the GSH and ophthalmic acid synthetic pathways, which agrees with the role of Nrf2 as an important transcriptional regulator of GCL and GS, both of which are involved in GSH and ophthalmic acid synthesis. However, as Nrf2 is involved in the regulation of a vast number of cell defence genes in addition to GCL and GS, other pathways in which these cytoprotective genes are involved in may be modulated differently in the $Nrf2^{(-/-)}$ animals in response to chemically-induced stress compared with the $Nrf2^{(+/+)}$. Further investigation in this area could therefore provide further insight into the role of Nrf2 in cell defence. Indeed, through a global proteomic approach, we have shown that in addition to cell defence proteins, those involved in the synthesis and metabolism of fatty acids and other lipids were markedly modulated in the liver of the $Nrf2^{(-/-)}$ mouse compared with its wild type counterpart (Kitteringham *et al.*, 2010).

Therefore, in conclusion the $Nrf2^{(-/-)}$ mouse provides an excellent model for exploring the toxicological and physiological response to chemical stress, since there are clear differences in the susceptibility of these animals to APAP hepatotoxicity, both following an acute dose and chronic administration. The results suggest that the consequences of Nrf2

deletion manifest at two levels: first, there is a constitutive loss of cellular defence, which is reflected by the enhanced sensitivity of the $Nrf2^{(-/-)}$ at early time points after APAP administration. Second, there is a clear evidence of an abrogated ability to initiate an adaptive up-regulation in cell defence, since at the later time points, the toxicological response is attenuated in $Nrf2^{(+/+)}$ mice compared with $Nrf2^{(-/-)}$. The chronic studies do, however, indicate that Nrf2 is not the sole arbiter of toxicological sensitivity since both $Nrf2^{(+/+)}$ and $Nrf2^{(-/-)}$ mice showed markedly reduced sensitivity to APAP hepatotoxicity following a pre-conditioning treatment regime with increasing doses of APAP. Thus, the $Nrf2^{(-/-)}$ mouse model has great potential value for exploring the drug-induced chemical toxicity in the liver and other organs, but equally may provide a rich source of candidate biomarker for both preclinical and translational application.

6.4 Is there a need for new biomarkers?

At present, several biomarkers are already used in research and in the clinic. However, the drive for the discovery and development of new biomarkers should still be encouraged as current ones do not fulfil all the attributes of an ideal biomarker. For example, although serum ALT has been historically used as a marker of liver injury, elevated levels of this protein have also been reported in conditions where no histopathological evidence of liver damage was observed (Edgar *et al.*, 1998, Muller *et al.*, 2000, Jackson *et al.*, 2008, Thulin *et al.*, 2008). As such, part of this PhD project was to investigate the GSH analogue, ophthalmic acid and its potential as a biomarker of oxidative stress.

Interest in ophthalmic acid has stemmed from findings by another group demonstrating significant elevations in liver and serum ophthalmic acid in parallel to a significant depletion in hepatic GSH in mice that were treated with either APAP, BSO or DEM (Soga *et al.*, 2006). Based on these results, this group concluded that this increase was a consequence of a combination of GSH and cysteine depletion in response to chemical stress. Subsequently, this group proposed ophthalmic acid as a potential biomarker of GSH consumption hence oxidative stress. Such a role could prove advantageous in research and in the clinic given the importance of GSH in redox homeostasis, cell defence and in the limitations of GSH itself as an index of oxidative stress. As such and as mentioned above, the purpose of the work discussed here was to further investigate this proposed function of ophthalmic acid particularly in its transferability across *in vitro* and *in vivo* experimental

systems. Furthermore, initial work was carried out, as described in chapter 5, to assess the potential of ophthalmic acid as a clinical biomarker as no amenable method of measuring hepatic GSH is currently available for clinical use.

Results from work carried out in chapter 4 on mice treated with APAP was in broad agreement with findings reported in the literature, as elevations in liver and serum ophthalmic acid were also observed in our study during which hepatic GSH levels decreased. Similar results were also observed in the $Nrf2^{(+/+)}$ and $Nrf2^{(-/-)}$ animals that were investigated in chapter 3 indicating the consistent response of ophthalmic acid to APAP-induced stress. However, in contrast to the literature, the observed increase in liver and serum ophthalmic acid both in chapters 3 and 4 were later than the immediate decrease in hepatic GSH, suggesting that ophthalmic acid is not a direct marker of GSH consumption. As discussed in chapter 4, this may have been due to several factors, in particular diet, as an important influencing factor as both GSH and ophthalmic acid rely on methionine, an essential amino acid, as source for cysteine and 2-AB, precursors in GSH and ophthalmic acid syntheses respectively. Animals used in our study were given free access to food whilst Soga and colleagues (2006) used starved animals in their study. As such and considering the importance of diet in both GSH and ophthalmic acid syntheses, the differences in experimental approach employed by both groups may have also led to the difference in results observed. However, regardless of these differences, as our results demonstrated that ophthalmic acid levels can be influenced by other factors other than GSH depletion, it can be concluded that ophthalmic acid does not directly reflect GSH consumption.

In support of this conclusion, work carried out in rats treated with DEM demonstrated a dose-dependent decrease in hepatic GSH but no significant increase in ophthalmic acid. *In vitro* work on the mouse cell line Hepa1c1c treated with NAPQI as well as the human THLE-Null and THLE-2E1 cell lines treated with APAP were also in agreement with this, as although GSH depletion was observed, no concurrent increase in ophthalmic acid took place. The disparity between the *in vivo* and *in vitro* results however could be due to intrinsic differences between the experimental models themselves. The increase in ophthalmic acid may rely on cell signalling between different cell types therefore limiting the use of cell lines in monitoring ophthalmic acid response to chemical stress. Ophthalmic acid could also be synthesised by a particular cell type therefore *in vitro* studies are dependent on identifying and using that particular cell type. Interestingly, the late increase in ophthalmic acid has been consistent with liver toxicity measured as an increase in serum

ALT activity *in vivo*. This correlation is worth investigating further especially as the exact role of ophthalmic acid basally or during chemical stress is yet unknown. On other hand, as ophthalmic acid synthesis relies on the activity of GCL, it is possible that the trigger that leads to the up-regulation of ophthalmic acid synthesis is the availability of the precursor cysteine. As discussed in chapter 4, GCL has a higher affinity for cysteine than 2-AB therefore it is possible that cysteine depletion is the trigger for the up-regulation of ophthalmic acid and not GSH. This could be further investigated by repeating the experiments discussed in the section with the addition of monitoring for cysteine levels.

In parallel to the work described in chapter 4 where the utility of ophthalmic acid as a marker of oxidative stress *in vivo* and *in vitro* was investigated, work described in chapter 5 was undertaken to further evaluate the breadth of utility of ophthalmic acid, in particular, for its potential use in a clinical setting. As such, preliminary work was carried out in chapter 5 where attempts were made to detect and quantify ophthalmic acid in the serum of healthy volunteers. The significance of this work is due to the fact that hepatic GSH in man is highly inaccessible. A marker that could directly indicate perturbation in the GSH content of the liver of an individual would therefore be an enormous advantage. Measurement of ophthalmic acid in serum was achieved only when using a highly sensitive mass spectrometer the QTRAP5500. Thus, whilst we have the capability to detect basal ophthalmic acid in human serum, a highly important factor in the development of a biomarker particularly for clinical use, this currently requires expensive, high specification instrumentation that will not be generally amenable. Serum ALT was used as a biomarker comparator in this study. From this, we have demonstrated that both ALT and ophthalmic acid are subject to intra- and inter-individual variation. Although only using a small cohort, this suggests that there will always be intrinsic intra- and inter-individual variability in ophthalmic acid level however, with regards to its development as a clinical biomarker, determination of a threshold that indicates its purpose is the key message, as determined for ALT using Hy's Law (Zimmerman, 1968, Zimmerman, 1978, Senior, 2009). Interestingly, one volunteer from whom only one ALT measurement was detected from all samples collected in the study also exhibited the highest level of ophthalmic acid in a different serum sample. This provides further support in the correlation previously observed between ophthalmic acid and ALT in chapter 5 and therefore merits further investigation.

6.5 Conclusion and future directions

The work carried out in this PhD project aimed to contribute to the effort in developing model systems to facilitate better research in drug safety. The THLE-2E1 cell line, the $Nrf2^{(-/-)}$ mouse and the GSH analogue ophthalmic acid all in their own right have the potential as useful tools in the study of chemical stress. However, as demonstrated in this project, the development of a model system or biomarker that bears all attributes required to directly reflect the fate of a drug in the human body to allow accurate prediction of its efficacy and safety is still a major challenge. Furthermore, even with the existence of excellent models of a particular health condition for example, caution should be taken when extrapolating results in the laboratory to the clinic as these are isolated systems and are therefore under conditions that are different in the human body.

Whilst the studies performed during this project have confirmed that each of the models investigated has potential for use in the preclinical evaluation of chemical stress, none provide all the attributes needed to address all toxicological issues. Thus, further developmental work is required to both identify new models and to enhance the application of existing ones. The procedures established during this course of my studies should help to provide a framework for the future optimisation of both preclinical and translational models for investigating drug-induced toxicity.

APPENDIX

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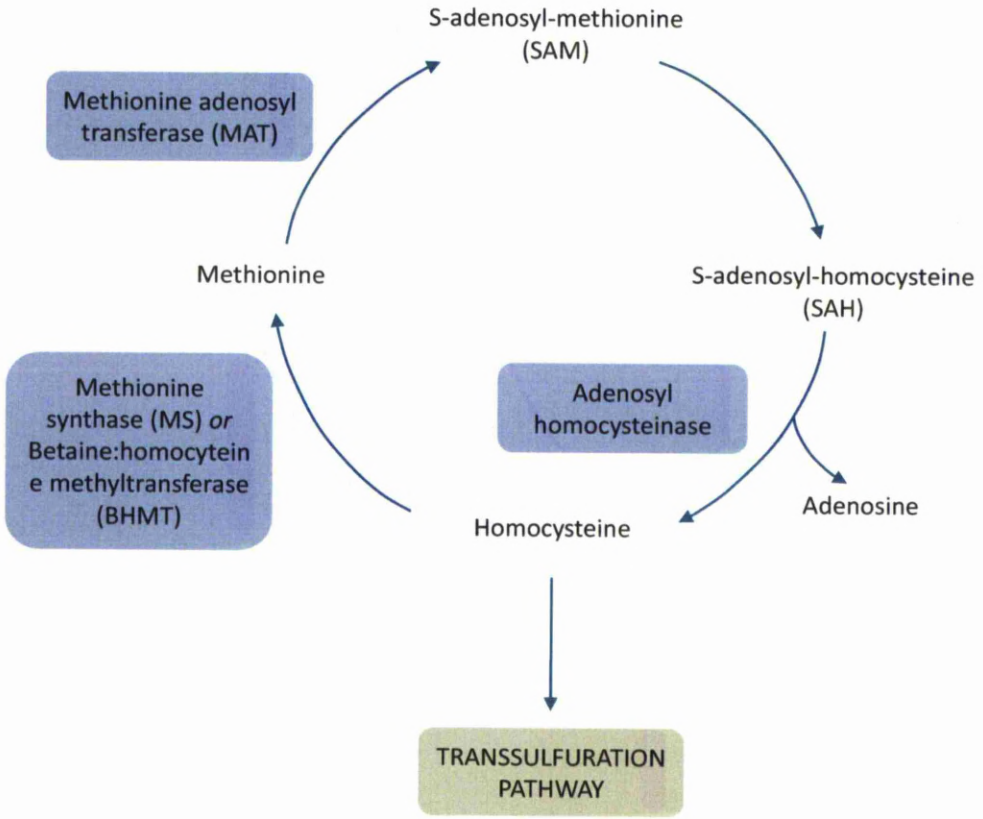
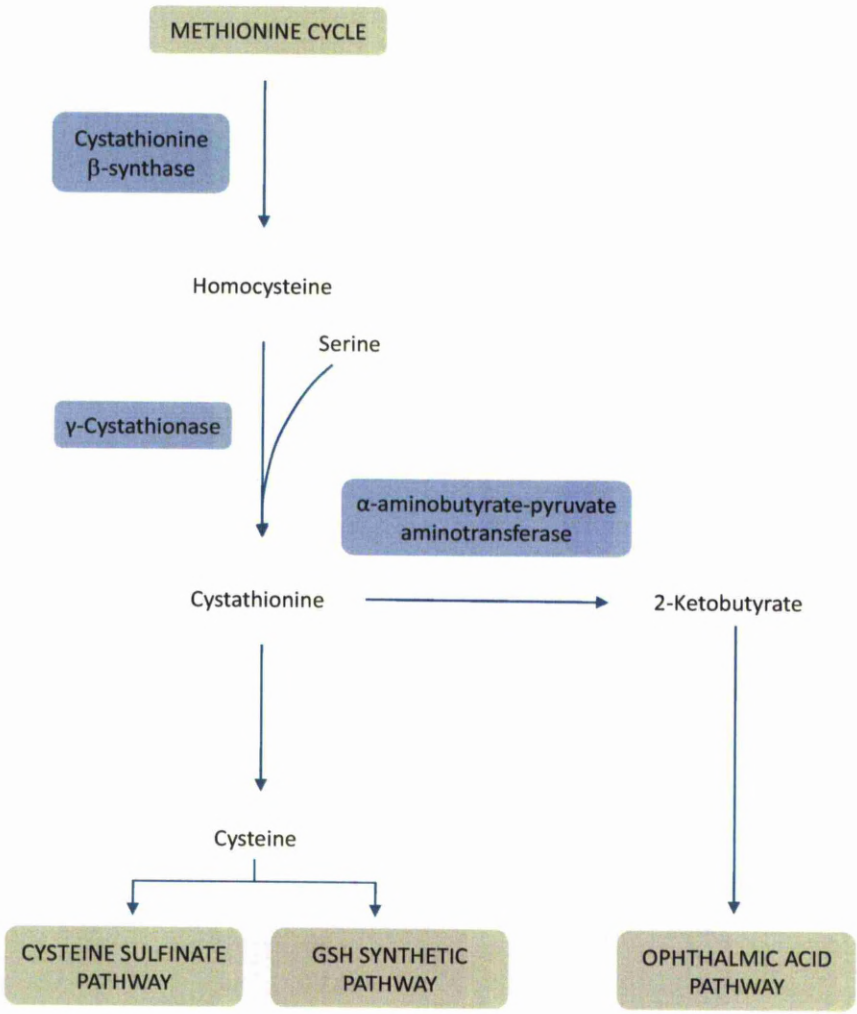
Appendix 1: The methionine cycle



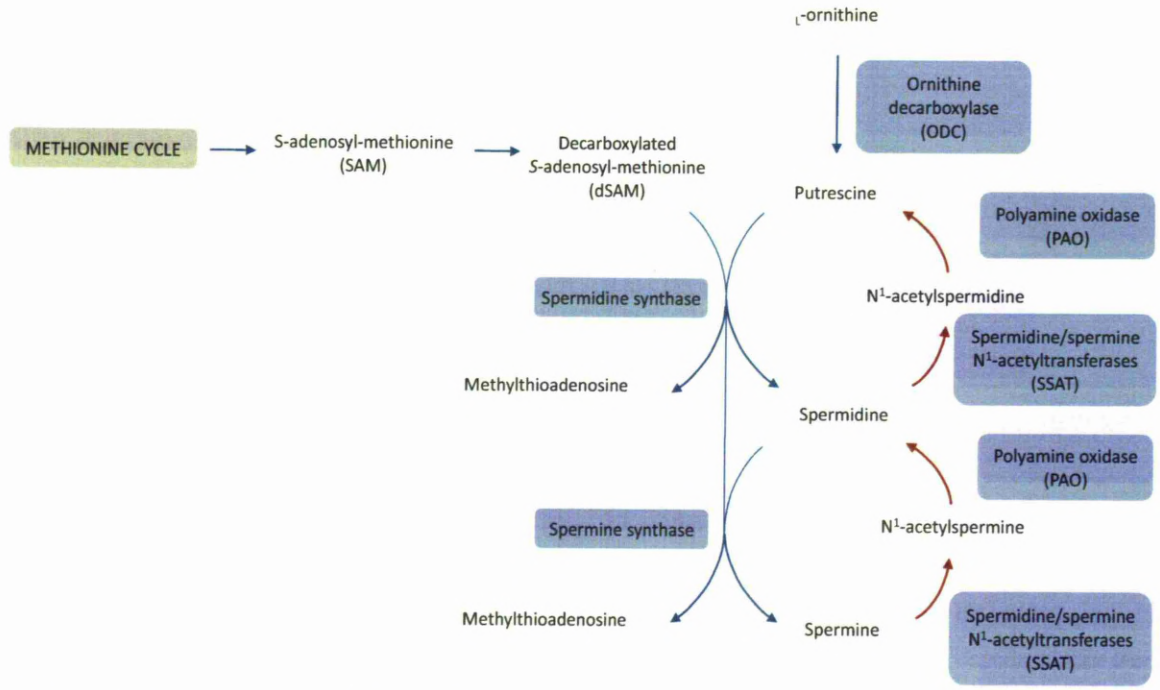
Appendix 1. The methionine cycle. Methionine is an essential sulphur-containing amino acid that is required in a variety of fundamental biological processes, particularly during protein synthesis. The methionine cycle comprises both trans- and remethylation reactions. Through the catalytic activity of methionine adenosyl transferase (MAT), methionine is converted to the active *S*-adenosyl methionine (SAM) which is capable of donating its methyl group to an acceptor to produce *S*-adenosyl homocysteine (SAH). SAH is then hydrolysed to homocysteine and adenosine by SAH hydrolase. At this point, homocysteine may be remethylated back to methionine by methionine synthase (MS) and/or by betaine:homocysteine methyltransferase (BHMT), where the entire methionine cycle starts again. Alternatively, homocysteine may be utilised as the initial substrate that enters the transsulfuration pathway (Figure 1.8), providing the link to GSH synthesis.

Appendix 2: The transsulfuration pathway



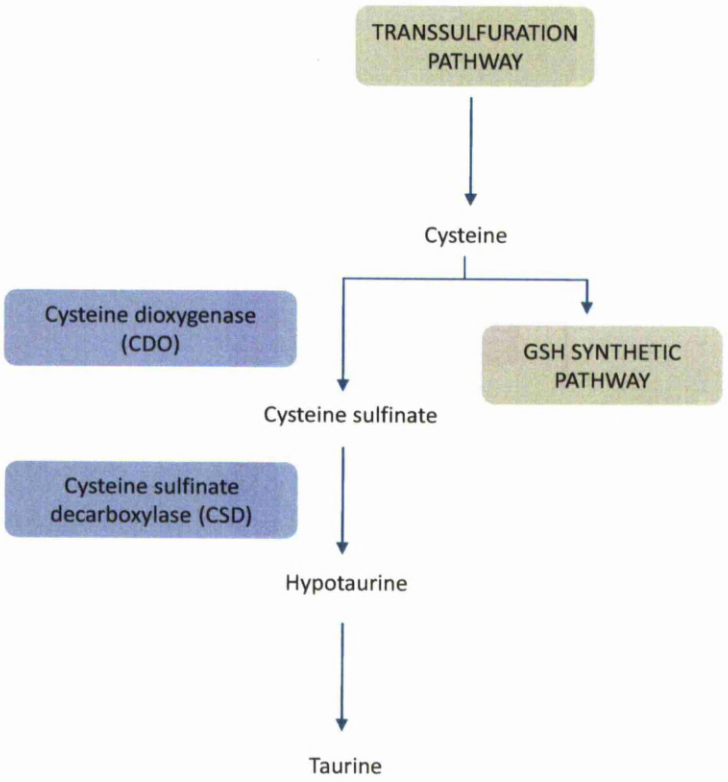
Appendix 2 The transsulfuration pathway. This is the major route of cysteine biosynthesis and is considered to be redox sensitive (Deplancke and Gaskins, 2002, Vitvitsky *et al.*, 2003, Banerjee and Zou, 2005). This pathway is initiated by a condensation reaction between homocysteine and serine to form cystathionine, catalysed by cystathionine β-synthase (CBS). Subsequent hydroxylation of cystathionine by γ-cystathionase (γ-Cys) leads to the production of cysteine and α-ketobutyrate. Cysteine may then be used as a substrate for GSH synthesis (Figure 1.5) amongst many other cellular activities in which it is involved (Jessop *et al.*, 2004), whilst α-ketobutyrate is utilised for ophthalmic acid synthesis (Figure 1.11) (Cliffe and Waley, 1958, Cliffe and Waley, 1961). At excess quantities, cysteine may undergo a series of oxidation reactions to form taurine or inorganic sulphates (Figure 1.10) (Stipanuk and Rotter, 1984, Bagley and Stipanuk, 1994, Bagley and Stipanuk, 1995, Bella and Stipanuk, 1995, Stipanuk, 2004, Stipanuk *et al.*, 2004, Dominy *et al.*, 2006).

Appendix 3: Polyamine metabolism



Appendix 3. Polyamine metabolism. Putrescine, spermine and spermidine are natural polyamines which are ubiquitously found in eukaryotic cells (Russell, 1980, Pegg and McCann, 1982). Putrescine is formed from the decarboxylation of ornithine through the activity of ornithine decarboxylase (ODC) (Tabor and Tabor, 1984, Casero and Pegg, 1993). Subsequent reaction of putrescine with decarboxylated SAM (dcSAM), a metabolic product of the methionine cycle (formed from the decarboxylation of SAM by SAM decarboxylase (SAMDC)), leads to the formation of spermidine and spermine by spermidine and spermine synthases respectively (Tabor and Tabor, 1984, Casero and Pegg, 1993). Conversely, spermidine/spermine *N*-acetyltransferases (SSAT), which are induced by both natural and synthetic polyamines as well as other toxicants, are responsible for the sequential acetylation of spermine to spermidine and eventually putrescine (Bolkenius and Seiler, 1981).

Appendix 4: The cysteine sulfinate pathway



Appendix 4. The cysteine sulfinate pathway. This is the primary source of taurine, through the oxidation of cysteine to cysteine sulfinate by cysteine dioxygenase (CDO) and the decarboxylation of cysteine sulfinate by cysteine sulfinate decarboxylase (CSD). This pathway depends upon the expression level and regulation of both CDO and CSD, as well as the availability of cysteine and methionine (Stipanuk and Rotter, 1984, Bagley and Stipanuk, 1994, Bagley and Stipanuk, 1995, Bella and Stipanuk, 1995, Stipanuk, 2004, Stipanuk *et al.*, 2004, Dominy *et al.*, 2006). Transport of taurine may also contribute to its intracellular concentration which may depend upon the expression level and regulation of a taurine transporter (TauT) as well as the extracellular concentration of this molecule. Taurine is present at high concentrations in the body (5 – 50 mM) and is implicated in a wide array of cytoprotective mechanisms including antioxidant effects, modulation of intracellular calcium level and ion channel activity as well as membrane and protein stabilisation (Geggel *et al.*, 1985, Rana and Sanders, 1986, Huxtable, 1992, Chen, 1993, Redmond *et al.*, 1996, Wang *et al.*, 1996, Waters *et al.*, 2001, McCarty, 2004, Manna *et al.*, 2008, Manna *et al.*, 2009).

Appendix 5. References for promising biomarkers of liver injury. Adapted from (Ozer *et al.*, 2008) and (Antoine *et al.*, 2009).

Biomarker	Reference
Malate dehydrogenase (MDH)	(Korsrud *et al.*, 1972, Zieve *et al.*, 1985, Kawai and Hosaki, 1990, Misra *et al.*, 1991, Zelewski and Swierczynski, 1991, Amacher *et al.*, 2005)
α-glutathione-S-transferase (GST-α)	(Beckett *et al.*, 1989, Giffen *et al.*, 2002, Coles and Kadlubar, 2005, Tong *et al.*, 2005) For polymorphic differences in humans: (Coles *et al.*, 2001)
Purine nucleotide phosphorylase (PNP)	(Ohuchi *et al.*, 1995, Mochida *et al.*, 1999, Fukuda *et al.*, 2004, Amacher *et al.*, 2005)
Paraoxonase 1 (PON-1)	(Feingold *et al.*, 1998, Rodrigo *et al.*, 2001, Ferre *et al.*, 2002, Meneses-Lorente *et al.*, 2004, Kilic *et al.*, 2005, Rozek *et al.*, 2005, Rozenberg *et al.*, 2005) For polymorphisms in human PON1 activity: (Mueller *et al.*, 1983, Davies *et al.*, 1996, Josse *et al.*, 1999, Richter and Furlong, 1999, Brophy *et al.*, 2001, Furlong *et al.*, 2002, Costa *et al.*, 2003a, Costa *et al.*, 2003b, Amacher *et al.*, 2005, Costa *et al.*, 2005)
Arginase 1	(Ashamiss *et al.*, 2004, Murayama *et al.*, 2007)
Serum F protein (4-hydroxyphenylpyruvate dioxygenase (HPD))	(Fravi and Lindenmann, 1968, Oliveira and Vindlacheruvu, 1987, Beckett *et al.*, 1989, Foster *et al.*, 1989, Callaghan *et al.*, 1994, Neve *et al.*, 2003)
Cytochrome c	(Hu *et al.*, 1999, Ben-Ari *et al.*, 2003, Adachi *et al.*, 2004, Kobayashi *et al.*, 2004b, Sakaida *et al.*, 2005, Miller *et al.*, 2008)
High mobility group box protein 1 (HMGB1)	(Wang *et al.*, 1999, Scaffidi *et al.*, 2002)
Ophthalmic acid	(Waley, 1953, Ballatori and Dutczak, 1994, Leslie *et al.*, 2001, Leslie *et al.*, 2003, Soga *et al.*, 2006)
Keratin-18 (K18) - fragmented	(Bantel *et al.*, 2004, Schutte *et al.*, 2004, Cummings *et al.*, 2008a, Cummings *et al.*, 2008b, Tao *et al.*, 2008)
Keratin-18 (K18) – full length	(Bantel *et al.*, 2004, Schutte *et al.*, 2004, Cummings *et al.*, 2008b)

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